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Characterization of a Decarboxylation - Dependent Transaminase and the Metabolism of Alpha-Aminoisobutyric Acid.

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CHARACTERIZATION OF A DECARBOXYLATION-DEPENDENT TRANSAMINASE AND THE METABOLISM OF ALPHA-AMINOISOBUTYRIC ACID

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LIST OF ABBREVIATIONS

Ac	acetate
AIB	∝-amino isobutyric acid
C	centigrade
cm	centimeter
Enz	enzyme: decarboxylation-dependent transaminase
g	gram
x g	gravity, centrifugal
v	initial velocity
ISPA	isopropylamine
l	liter
MeOH	methanol
MeAc	methyl acetate
Km	Michaelis constant
µg	microgram
µl	microliter
umoles	micromoles
mg	milligram
ml	milliliter
mM	millimolar
min	minute
M	molar
nm	nanometer
OD	optical density
%	percent

PL	pyridoxal
PLP	pyridoxal-5'-phosphate
PM	pyridoxamine
PMP	pyridoxamine-5'-phosphate
Py	pyruvate
Py-ald	pyruvic aldehyde
rpm	revolutions per minute
S	substrate concentration
TCA	trichloroacetic acid
v/v	volume per volume
w/v	weight per volume

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ABSTRACT

The growth of a soil pseudomonad in a medium containing ∝-amino isobutyric acid as sole carbon source was investigated. Growth appeared to be limited either by failure of the medium to satisfy nutritional requirements or by the production of some toxic products by the organism during growth. The precise cause or causes remained undetermined.

The decarboxylation-dependent transaminase involved in the degradation of ∝-amino isobutyric acid was partially purified and the reaction mechanism characterized. The enzyme was purified 65-fold by combining protamine sulfate treatment, heat treatment, ammonium sulfate and acetone fractionation, and Sephadex gel filtration.

The decarboxylation-dependent transaminase required stoichiometric amounts of pyruvate and catalytic amounts of pyridoxal-5'-phosphate for activity. The resolved enzyme was reactivated with either pyridoxamine-5'-phosphate or pyridoxal-5'-phosphate. The coenzyme was easily resolved from the holoenzyme by dialysis in the presence of the substrate. That pyridoxal-5'-phosphate participated in the enzymatic reaction as opposed to being a structural component of the enzyme was determined by sucrose density gra-

dient centrifugation of holo- and apoenzyme. Both enzyme forms sedimented at equal rates. Results obtained with $NaBH_4$ reduction of holo- and apoenzyme again indicated an active catalytic role for pyridoxal-5'-phosphate. Reduction of holoenzyme resulted in irreversible inactivation, but reduction of apoenzyme had little effect on the enzyme.

Neither acetone nor isopropylamine inhibited the reaction and the enzyme did not possess any isopropylamine-pyruvate transamination activity. Stoichiometric amounts of pyridoxal-5'-phosphate or pyridoxal did not replace pyruvate even in the presence of catalytic amounts of the keto acid acceptor. Transamination between pyridoxamine-5'-phosphate and pyruvate was not catalyzed by the enzyme nor did the enzyme in the pyridoxamine-5'-phosphate form transaminate with pyruvate. The enzyme appeared to be an allosteric enzyme with ∝-amino isobutyric acid and pyruvate as modulators.

Manometric experiments with adapted and non-adapted whole cells indicated acetone, acetol, pyruvic aldehyde and methyl acetate as possible intermediates in the oxidation of ∝-amino isobutyric acid.

INTRODUCTION

The resistance to metabolic attack exhibited by α-dialkyl amino acids has been attributed to the absence of the α hydrogen atom (12). α-Methyl substituted amino acids have been used as inhibitors of PLP-dependent enzymes (72, 74) and as metabolic tracers in the study of amino acid transport (12).

Aaslestad and Larson (3) reported the isolation of a soil pseudomonad capable of utilizing AIB as its sole carbon source. They were the first to partially purify an enzyme capable of degrading AIB. The reaction catalyzed by the enzyme was found to be dependent upon the presence of PLP and pyruvate. The products of AIB catabolism were found to be acetone, CO_2 and alanine. On the basis of the stoichiometry of the reaction these workers termed the enzyme a decarboxylation-dependent transaminase as proposed by Kalyankar and Snell (37) for a similar reaction occurring in PL model system. The enzyme was also shown to decarboxylate the D and L isomers of isovaline. Bailey and Dempsey (4) have also purified a decarboxylation-dependent transaminase. The enzyme was also dependent upon the presence of PLP and pyruvate for catalytic activity and degraded AIB and L-isovaline but not D-isovaline (5).

The purpose of the research reported in this dissertation was to purify further and characterize the decarboxylation-dependent transaminase, to determine the role of the coenzyme in the reaction and to determine a pathway for the degradation of AIB.

REVIEW OF LITERATURE

α-Amino acids possessing two alkyl groups in the α position are structurally similar to natural occurring amino acids. In general, the substitution of the α hydrogen by an alkyl group makes the amino acid inert in many enzymatic reactions. The most common examples of α-dialkyl amino acids studied in biological systems are α-aminoisobutyric acid (2-methylalanine) and isovaline (2-methyl-2-aminobutyric acid).

The metabolic inertness of these compounds was first reported in 1937 (40). It was found that these amino acids were excreted unchanged in the urine after subcutaneous administration into dogs. Christensen, Aspen and Rice in 1956 (11) reported that these compounds were concentrated by the liver in rats after intraperitoneal injection. Noall et al. (55) demonstrated that 2-methylalanine-1-C^{14} was not incorporated into protein in a mammalian system.

The resistance of α-dialkyl amino acids to degradation by cell-free systems has been reported (28). α-Methylserine and α-amino-β-phenoxyisobutyric acid are not oxidized by cottonmouth snake L-amino acid oxidase or kidney D-amino acid oxidase. Likewise, Thibert et al. (72) reported that α-alkyl substituted cystines were not cleaved by the PLP-dependent enzyme,

cystathionase. The same amino acid derivatives were not modified in a PLP non-enzymatic model system.

Since α-dialkyl amino acids appeared to be resistant to metabolic attack, at least in mammalian systems, they have often been employed as metabolic tracers. Christensen et al. (12) followed the accumulation of AIB and isovaline in Ehrlich mouse ascites carcinoma cells. Employing the same cells Christensen and Riggs (13) later reported that these amino acids were concentrated to a greater extent than their naturally occurring analogs. Furthermore, Noall et al. (55) and Hall (30) utilized the metabolic resistance of AIB in a study of the endocrine control of cell permeability to amino acids. Studies on amino acid transport using AIB have also been carried out in microorganisms by Drapeau and MacLeod in 1963 (20), and Marquis and Gerhardt in 1964 (48).

α-Dialkyl amino acids have been found to inhibit transaminases (7), amino acid decarboxylases (15, 57, 68), and D-amino acid oxidases (56). β-Eliminations such as those catalyzed by cystathionase are also inhibited by α-alkyl derivatives of cystine (72).

Although a resistance to metabolic attack is a prominent feature of α-alkyl substituted amino acids, there are some research reports which have demonstra-

ted susceptibility of these amino acids to enzymatic and non-enzymatic reactions. As early as 1908 Ehrlich (23) observed that yeast utilized the L-isomer of DL-isovaline and he isolated the D-isomer from the spent culture fluid. In 1926 den Dooren de Jong (19) reported on the utilization of AIB and isovaline as nitrogen sources by bacteria. Christensen and Jones (14) reported that a bacterial cell extract from the intestinal tract of mice released $C^{14}O_2$ and ammonia from AIB-1-C^{14} but no definitive study was made. Weissbach, Lovenberg and Undenfriend (78) reported that mammalian amino acid decarboxylases acted on the L-isomers of 2-methyltryptophan, 2-methyl-5-hydroxytryptophan and 2-methyldihydroxyphenylalanine. Carlsson and Lindqvist (10) also reported the in vivo decarboxylation of 2-methyldihydroxyphenylalanine and 2-methylmetatyrosine in mice and rabbits. Hayaishi et al. (31) reported that AIB was transaminated at 4% of the rate of β-alanine by an α-alanine-β-alanine transaminase purified from Pseudomonas fluorescens. In non-enzymatic model systems using PL as a catalyst, Longenecker et al. (42) showed the cleavage of α-methylserine and α-hydroxymethylserine. Subsequently Wilson and Snell (79) reported the enzymatic degradation of these compounds by a soil pseudomonad. The

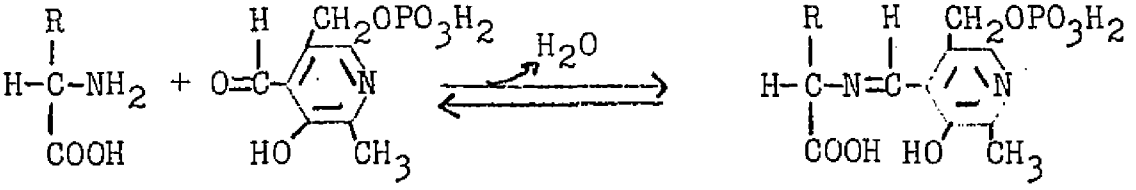
degradation of AIB has also been shown to occur in PL model systems. Kalyankar and Snell (37) demonstrated the non-enzymatic degradation of AIB by two different pathways. In the presence of PL, AIB was decarboxylated with the formation of CO_2 and ISPA or underwent a decarboxylation-dependent transamination reaction with the production of CO_2, acetone and PM.

Unsuccessful attempts have been made to show the direct enzymatic decarboxylation of AIB to CO_2 and ISPA (17). On the other hand the enzymatic degradation of AIB via the decarboxylation-dependent transamination reaction of the model system was first elucidated by Aaslestad and Larson (3). They partially purified and characterized an inducible decarboxylation-dependent transaminase from a soil pseudomonad which could grow on AIB as its sole carbon source. Whole cells were also found to oxidize D and L-isovaline at equal rates. However, the enzyme in the crude and partially purified states possessed nearly equal activity toward AIB, DL-isovaline and L-isovaline, but the activity with D-isovaline was only one-fourth of that with the other substrates. The Km for D-isovaline was smaller than for L and DL-isovaline, but very close to that of AIB (2). Bailey and Dempsey (4) subsequently characterized a decarboxylation-

dependent transaminase from a soil organism grown on glucose as the main carbon source and DL-isovaline as the nitrogen source. The enzyme decarboxylated L-isovaline, AIB and cycloleucine. However, decarboxylation of the D-isomer proceeded slowly and required stoichiometric amounts of enzyme (5).

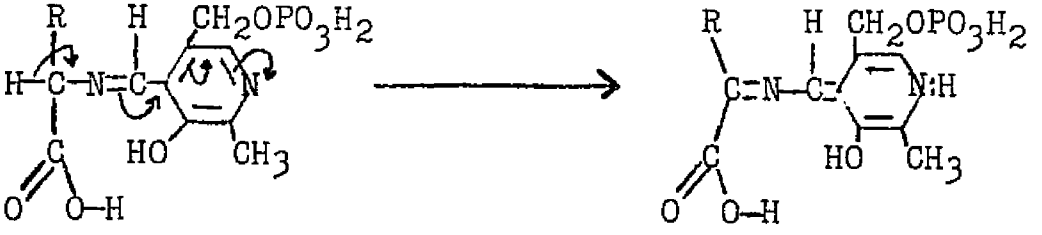
PLP is involved in all the reported enzymes and model systems which result in the degradation of α-dialkyl amino acids. Furthermore, PLP is involved in non-oxidative deamination, transamination and decarboxylation and it must be considered in any study of amino acid degradation. With one exception all transaminases studied thus far require PLP as a cofactor. Pyridoxamine-pyruvate transaminase which catalyzes the transamination between PM and pyruvate does not require PLP (18). With less efficiency, glutamic-oxaloacetic apotransaminase catalyzes a similar reaction between PM and α-ketoglutarate or oxaloacetate (76). The reaction mechanism for enzymatic transamination has been studied in detail and it appears to be similar to the non-enzymatic mechanism first proposed by Snell (64). The mechanism of the model system involves the reversible transfer of the amino group from the amino acid donor to the aldehyde group of PL. Schlenk and Fischer (61) discovered that in purified

glutamic-aspartic transaminase the vitamin B_6 content of the enzyme increased with the purity of the preparation. They then suggested the same mechanism for enzymatic transamination reaction as proposed for the model system assuming the formation of an azomethine linkage intermediate between the protein bound coenzyme and the amino acid as shown below (62):



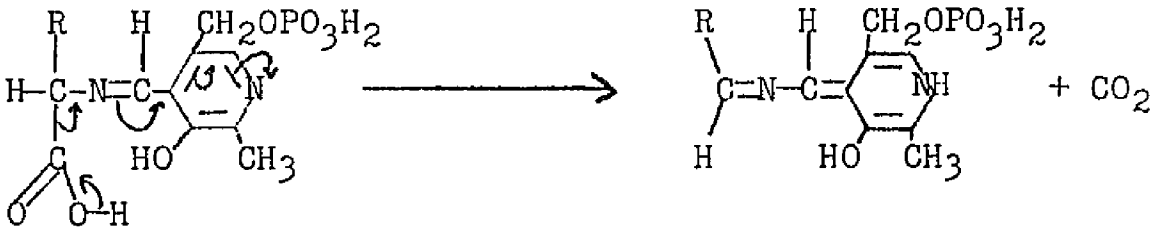
The imine or Schiff base formed between the amino acid and PLP gives rise to a planar system of conjugated double bonds. This stable intermediate provides a mechanism for the displacement of electron pairs from the α carbon which can result in racemization, decarboxylation or transamination.

The Schiff base can isomerize by proton transfer of the α hydrogen or by decarboxylation to form an isomeric imine as shown below:



Proton transfer

Isomeric imine



Decarboxylation Isomeric imine

Hydrolysis of isomeric imines results either in the formation of an α-keto acid and PMP or an amine and PLP. Metzler et al. (51) and Braunstein (8) independently proposed the tautomerization of the azomethine linkage to explain results obtained with model systems. The formation of isomeric imines could be involved in the mechanism of action of many PLP-dependent enzymes.

Confirmatory evidence for Schiff base formation between PLP and amino acids has been obtained for non-enzymatic systems. Heyl et al. (33) coupled PL with amines with the formation of yellow Schiff bases which were then hydrogenated to form PM derivatives. These compounds were biologically inactive in nutritional studies with microorganisms (65) but some derivatives exhibited biological activity equal to pyridoxine in rats (32). Matsuo (50) carried out spectrophotometric studies on the reaction of amino acids with PLP. His work provided evidence for PLP-amino acid Schiff bases. He studied the non-enzymatic transamination

between PM and ∝-keto acids as well as transamination between amino acids and PL. His results provided additional evidence for the tautomerization of the aldimine and ketimine forms of the Schiff bases (49).

Metzler (52) performed spectrophotometric studies to determine equilibrium constants of the imines formed between PL and amino acids. His results showed that the imines were formed very rapidly and that the limiting step in the non-enzymatic transamination was the tautomerization of the imines. He extended his results to enzymatic transamination and concluded that the apoprotein provided the proper orientation for maximum tautomerization rates.

Chemical evidence for the formation of a Schiff base between enzyme and substrate has also been provided by $NaBH_4$ reduction of the imine intermediate. Preliminary experiments by Katunuma et al. (38) indicated that pyridoxyl-alanine, the substrate coenzyme intermediate of glutamic-pyruvate transaminase, could be identified by $NaBH_4$ reduction. Malakhova and Torchinsky (46) were able to isolate a substrate-coenzyme intermediate by reducing ∝-methylaspartate to aspartic-glutamic transaminase holoenzyme, and Buffoni (9) isolated the PL derivative of histamine-C^{14} from reduced histaminase-substrate complex.

The isomeric imine formation after removal of the α hydrogen is an important intermediate step in PLP-dependent reactions. In racemases the intermediate reacts monostereospecifically with a proton to form an amino acid of opposite configuration (39). In the case of threonine aldolase, the intermediate is glycine-pyridoximine and this reacts with acetaldehyde to form threonine (45). Glycine-pyridoximine is also the isomeric imine intermediate in the reaction catalyzed by serine transhydroxymethylase. This conjugate system can react with N^5,N^{10}-methylenetetrahydrofolic acid to form serine (60). It is important to note that the cleavage of serine by transhydroxymethylase is not affected by substitution of the α hydrogen by a methyl group (60), an observation which is consistent with the proposed mechanism for the non-enzymatic cleavage of β-hydroxy-amino acids by PL (51). The mechanism of α-β elimination reactions of amino acids can also be explained by the formation of an isomeric imine. It has been shown that decarboxylation of amino acids involved isomeric imine intermediates with PLP and in this case the α hydrogen is unaffected. Mandeles et al. (47) provided evidence for this mechanism by carrying out enzymatic decarboxylation of lysine, tyrosine and glutamic acid in D_2O. They found no

deuterium in the amines formed. From the imine intermediate an amine is formed by proton transfer with subsequent hydrolysis of the new imine.

In general, the mechanism of action of PLP-dependent enzymes involves the formation of a system of π electrons as a result of Schiff base formation between PLP and the amino acid. This conjugated system is extended by loss of a group from the α carbon in the formation of the isomeric imine intermediate. The free pair of electrons are then delocalized through the pyridine ring which stabilizes the intermediate. Furthermore, Dunathan (21) has suggested that the spatial configuration determines which group of the αcarbon will be released. The bond subject to attack should be in the plane perpendicular to the Schiff base system. The binding of PLP to the apoenzyme as well as the primary structure around the active site would determine which bond will be affected.

The mode of binding PLP to the apoenzyme is very important in determining the specificity and mechanism of action of PLP-dependent enzymes. Jenkins and Sizer (36) first proposed that the aldehyde group of the coenzyme was bound to the apoprotein from the spectral evidence obtained with purified glutamic-aspartic transaminase holoenzymes. Jencks and Cordes (35)

later showed that the imine form of PLP reacts more readily with carbonyl reagents. Following Jencks and Cordes report, PLP has been found to bind to the apoenzyme in the form of a Schiff base to several PLP-dependent enzymes (24).

Sodium borohydride reduction of numerous PLP-dependent holoenzymes has provided for the isolation of pyridoxyl-lysine residues (24). This technique has also been used in conjunction with selective hydrolysis to identify the amino acid sequence of the active site of PLP enzymes (34, 54, 70). It is of interest to note that pyridoxamine-pyruvate transaminase even though it does not require PLP for activity binds the substrate, pyridoxal, to lysine by formation of a Schiff base (18). The mechanism of action of this enzyme is considered to be the same as for any PLP-dependent enzyme. The enzyme uses pyridoxal as a substrate instead of a coenzyme.

The imine linkage between PLP and the epsilon amino group of lysine offers obvious advantages for enzymatic action. The Schiff base covalent bond tightly binds the coenzyme to the apoenzyme which prevents removal from the active site and maintains the enzyme in an active form.

Review articles on the mechanism of action of

pyridoxal phosphate have been presented by Braunstein (8) and more recently by Fasella (24). Two international symposia on pyridoxal phosphate catalysts are also available; one was held in Rome in 1962 (66), and the most recent one met in Moscow in 1966 (67).

MATERIALS AND METHODS

Chemicals

All inorganic salts were of analytical reagent grade. The buffer system (buffer A) contained 0.05 M potassium phosphate and 0.2 M KCl (pH 7.5).

AIB, alanine, and PMP were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. PLP was obtained from Calbiochem, Los Angeles, California. Pyridoxine and pyridoxal were obtained from Eastman Kodak, Co., Rochester, New York, and General Biochemicals, Chagrin Falls, Ohio. Pyruvic acid was obtained from Sigma Chemical Co., St. Louis, Missouri.

Carbon sources, for growth and induction experiments, such as glucose, glycerol, and nutrient broth were obtained from Allied Chemical, Morristown, New Jersey, Mallinckrodt Chemical Works, St. Louis, Missouri and Difco Laboratories, Detroit, Michigan respectively.

The sources of the suspected intermediates in the catabolism of AIB were obtained from the following companies: acetone (J. T. Baker Chemical Co., Phillipsburg, New Jersey), acetol and pyruvic aldehyde (Aldrich Chemical Co., Milwaukee, Wisconsin), methanol and sodium acetate (Mallinckrodt Chemical Works, St. Louis, Missouri), formaldehyde (Allied Chemical Company,

Morristown, New Jersey) and sodium formate (Merck and Company, Inc., Rahway, New Jersey).

Ninhydrin, 2,4-dinitrophenylhydrazine and sodium borohydride were obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, Eastman, Kodak Co., Rochester, New York and Matheson, Coleman and Bell, Cincinnati, Ohio, respectively. Sephadex G-200 was purchased from Pharmacia Fine Chemical Inc., Piscataway, New Jersey.

Carbon tetrachloride, chloramphenicol and TCA were bought from Matheson, Coleman and Bell, Cincinnati, Ohio, Calbiochem, Los Angeles, California and Mallinckrodt Chemical Works, St. Louis, Missouri, respectively.

Organism

The Pseudomonas sp. used for this study was isolated from soil by Aaslestad and Larson (3) using elective culturing techniques. The enrichment medium contained 0.2% AIB in the salts mixture of Stanier (69) adjusted to pH 7. The organism was maintained by successive transfers in screw cap tubes on the same medium solidified with 2% agar. Cultures were transferred approximately every two weeks and periodically examined microscopically for evidence of contamination.

Culturing the Organism

Cells used to study the decarboxylation-dependent transaminase were grown in a New Brunswick Continuous Culture Apparatus Model CF500. The inoculum for the continuous culture was prepared as follows: Cells from two slant cultures were suspended in 5 ml of buffer A and this was employed to inoculate two Roux flasks containing 125 ml of AIB agar medium. The Roux flasks were incubated at 30 C for three days. The cells from the Roux flasks were suspended in 10 ml of buffer A and used to inoculate two 2-liter flasks containing 1000 ml of AIB broth medium. The flasks were placed on a gyrorotary shaker at room temperature for four days and the contents used to inoculate a carboy containing 10 l of medium. The contents of the carboy were aseptically siphoned into the sterile continuous culture vessel. The culture vessels were autoclaved for one hour at 121 C prior to use. The stainless steel-glass wool air filter, siphon tubes and connecting tygon tubes were autoclaved for 15 minutes at 121 C.

The organism was grown in the culture apparatus under continuous culture conditions. The temperature of the culture vessel was held at 30C while the contents were agitated (200 rpm) and aerated (approxi-

mately 14 l of air/min.).

Centrifugation of large volumes of culture from the continuous culture apparatus was accomplished by use of a Sharples steam-driven continuous centrifuge. The harvested cells were washed once in distilled water and once in buffer A employing a Sorvall Model RC-2 centrifuge (Ivan Sorvall, Inc., Norwalk, Connecticut) at 16,300 x g for 15 minutes.

The cell paste was used immediately or stored in capped tubes at -23 C. The yield, in terms of wet weight of cells, was on the order of 0.6 to 0.8 g/l..

Preparation of Cellular Extracts

Cellular extracts were prepared from either fresh or frozen washed cells. The bacterial cell paste was suspended in a small volume of buffer A to give a thick suspension and chilled to approximately 5 C. The chilled slurry of bacteria was then poured into a Rosett cooling cell (58) and placed in an ethylene glycol-water bath at -5 C. The bacteria were subjected to disruption by the use of a 20Kc Bronson sonifier for five to ten minutes depending on the volume of the slurry. The temperature of the bacterial suspension remained at 10^{+}_{-} 2 C during sonication. Unbroken cells and cellular debris were removed by centrifugation for 30 minutes at 27,000 x g in a Sorvall RC2 centrifuge

at 4 C. The clear cellular extract was decanted and used for experimentation.

Manometric Methods

O_2 consumption and CO_2 evolution were measured manometrically with a Warburg respirometer by procedures outlined by Umbreit, Burris and Stauffer (73). For studies involving O_2 uptake the Warburg vessel contained 0.5 ml of Buffer A in the main compartment and 0.2 ml of 20% KOH in the center well. Substrates were placed in the experimental but not in the control vessel. A suspension of washed bacterial cells, standardized by OD at 600 nm in a Bausch and Laumb Spectronic 20, for which the dry weight was determined from a standard curve was added to one of the side arms. Sufficient distilled H_2O was used to bring the volume of fluid in each vessel to a final volume of 3 ml after all other additions had been made. Oxidation studies were carried out at 30 C with air as the gaseous phase.

For studies involving CO_2 evolution, KOH was omitted from the system and 0.2 ml of 20% TCA was added to one of the side arms. The other side arm contained cellular extract while the main compartment contained substrate, and any other additions such as coenzyme, inhibitors and buffer. The endogenous production of

CO_2 was measured in a vessel with no substrate. The reaction was initiated by tipping the cellular extract from the side arm into the main compartment. The reaction at the end of the incubation period and CO_2 was quantitatively released by the addition of TCA from the side arm of the vessel.

Each Warburg vessel and manometer was calibrated for K_{O_2} and K_{CO_2} values at 30 C and a 3 ml final volume of fluid.

Assays

The decarboxylation-dependent transaminase was quantitatively assayed either manometrically by measuring the CO_2 released or by measuring acetone production by the 2,4-dinitrophenylhydrazine method of Greenberg and Lester (29) as follows: The enzyme preparation was added to a reaction vessel containing substrates, coenzyme and buffer (13 μmoles/ml phosphate pH 7.8). The reaction mixture was then allowed to incubate at 30 C for a predetermined period of time. An aliquot of 0.1 ml from the mixture was added to screw cap tubes containing 2 ml of 0.1 M 2,4-dinitrophenylhydrazine in 2 N HCl plus 2.9 ml of distilled H_2O. Two ml of carbon tetrachloride was added and the dinitrophenylhydrazone of acetone was extracted with shaking on a mechanical gyrorotary shaker for 15 minutes. The

aqueous layer was discarded and the carbon tetrachloride layer was washed twice with 10 ml of distilled water. The carbon tetrachloride was extracted with shaking for three minutes with three ml of 0.5 M NaOH. The water layer was then discarded and the OD of the carbon tetrachloride layer was determined in a Beckman DB spectrophotometer at 420 nm. The umoles of acetone produced in the reaction was determined from a standard curve obtained using the same analytical procedure with measured amounts of acetone.

Specific activity for the decarboxylation-dependent transaminase was expressed as umoles of product formed, CO_2 or acetone, per mg of protein per minute.

Preliminary protein determinations were carried out by measuring OD at 280 and 260 nm according to the method of Warburg and Christian (77). Protein determination by the method of Lowry et al. (43) was used when final specific activities were calculated.

Alanine production catalyzed by enzyme preparations was qualitatively detected by thin layer chromatography. Samples were applied to precoated silica gel G plates and chromatographed using n-propanol and water (1:1 v/v) as solvent (75). The alanine spots were detected by spraying the dried plates with a 0.15% (w/v) solution of ninhydrin in acetone and heating the plates at 90 C for 5 minutes.

Enzyme Purification

The procedure for purification of decarboxylation-dependent transaminase was performed, in part, as described by Aaslestad (1) with additional steps to provide for further purification. The cellular extract was prepared as described in the previous section. For each 100 mg of protein in the crude extract, 12 mg of protamine sulfate was added slowly using a 1% solution in 1 M phosphate buffer pH 7. The protamine sulfate-treated extract was allowed to stand in the cold for one hour and the precipitate was sedimented at 27,000 x g for 30 minutes in a refrigerated centrifuge. To the supernatant fluid, KCl and PLP (0.5 M and 10^{-4} M final concentration, respectively) were added. The supernatant fluid was heated to 50 C for five minutes and clarified by centrifugation at 27,000 x g. The sediment was discarded and the supernatant fluid was adjusted to 10 mg of protein/ml using 1 M PO_4 buffer (pH 7.5) by the spectrophotomeric method. Solid $(NH_4)_2SO_4$ was then added to the supernatant fluid until 45% of saturation was reached. The resulting precipitate was collected by centrifugation and was dissolved in a minimal amount of buffer A. The enzyme solution was dialyzed against buffer A for 4 hours. The protein concentration was adjusted again

to 10 mg/ml using 1 M phosphate buffer (pH 7.5) and further $(NH_4)_2SO_4$ fractionation (0-20 and 20-60% of saturation) was carried out. The 20-60% precipitate was retained, redissolved in a minimal amount of buffer A and dialyzed against the same buffer for 4 hours. Sequential fractionation at room temperature was then performed using acetone at v/v ratio of 0.10, 0.25, 0.50, 0.75 and 1.00. Each precipitated fraction was removed by centrifugation, redissolved in a minimal amount of buffer A and dialyzed at 4 C overnight. The enzyme was found in the 0.50-0.75 fraction. Subsequently the enzyme was applied to a G-200 Sephadex column previously equilibrated with buffer A and active fractions were collected, pooled and concentrated by dialysis against solid sucrose.

Enzyme Resolution

In order to remove PLP from the decarboxylation-dependent transaminase the enzyme preparations were dialyzed in the presence of 0.2% AIB in buffer A. After dialysis the enzyme was assayed in the presence and absence of the coenzyme. The percent resolution was determined by the following formula:

$$R = \frac{A - a}{A} \times 100$$

where R = % resolution
A = activity in the presence of PLP
a = activity in the absence of PLP.

Analytical Gel Electrophoresis

Procedures for disc eletrophoresis were those described by Davis (16). A sample gel was not used but all samples were concentrated in sucrose and were placed directly over the stacking gel. A Beckman Spinco Constat regulated power supply was used to supply 4 milliamps per tube and the current was applied for 45 to 60 minutes. Bromophenol blue was used as a tracking dye and Buffalo Black NBR NO. 6469 was used for gel staining. Staining was for 10 minutes followed by several changes of 7% acetic acid for destaining.

RESULTS

Growth of the Organism

The organism used in this study exhibited poor growth in the AIB medium. Aaslestad (1) reported that the minimal medium supplemented with 0.005% yeast extract plus 0.005% sodium acetate slightly increased the cell yield without drastically affecting the ability of the organism to oxidize AIB. On the other hand, higher concentrations of the supplements completely repressed the formation of the decarboxylation-dependent transaminase. As the supplemented medium did not show an appreciable increase in cell yield and higher concentrations of supplements depressed the formation of decarboxylation-dependent transaminase, one may speculate that the supplements were only providing growth material to increase the inoculum size. Therefore, the growth of the organism in the AIB medium was further investigated to determine the factor or factors responsible for its slow growth rate.

The organism was inoculated into two-liter flasks containing one liter of AIB minimal medium. The flasks were placed on rotatory shakers and the bacterial growth was followed spectrophotometrically with a BL spectrophotometer at 600 nm. Viable counts were made by taking samples from the growing culture, making appro-

priate dilution in sterile water, and plating out 0.1 ml of the diluted samples onto nutrient agar plates. The plates were incubated at 30 C for 24 to 48 hours and the isolated colonies were counted. The bacterium exhibited the characteristic lag and log phases of growth. The maximal viable count at the end of the log phase was around 3.7×10^7 cells/ml corresponding to an OD reading of 0.07 units. The end of the log phase was reached in 20 to 24 hours depending on the inoculum size. The average generation time was found to be 2.5 hours. The organism exhibited a long stationary phase, as determined by OD and showed no evidence of cell lysis. On the other hand, the stationary phase as determined by viable counts was very short. Furthermore, the stationary phase was followed by a logarithmic death phase. Figure 1 represents two different growth curves of the bacteria in AIB medium.

The small number of cells at the end of the logarithmic phase, as well as the short stationary phase and the logarithmic death phase, was indicative of substrate limitation and accumulation of toxic materials in the medium, or both. Therefore, the effect of substrate concentration on the growth rate of the organism was determined. For this experiment, one ml of a stan-

Figure 1. Growth curves of the organism in AIB minimal medium. The bacteria were grown on 2 liter flasks containing 1 liter of AIB minimal medium. Viable counts (○) were obtained by plating samples on nutrient agar and the OD (●) was obtained at 600 nm.

Log No. of cells
Log No. of cells
TIME (hrs)
G
G

dard inoculum (OD = 0.1) was used to inoculate a series of 100-ml flasks containing 50 ml of Stanier's salts supplemented with 0.005% yeast extract and varying concentrations of AIB. The flasks were placed on gyrorotary shakers and incubated at room temperature for 72 hours. At the end of the incubation period the OD was determined at 600 nm. The results of this experiment are shown in Figures 2 and 3. A linear correlation between optical density and substrate concentration was observed up to 0.1% AIB. However, substrate concentrations of 0.2% or higher, showed no evidence of such correlation. The result of this experiment indicated that 0.2% AIB was not substrate-limiting, and did not account for the logarithmic death phase.

Similar experiments were performed maintaining the concentration of AIB and yeast extract constant, while varying the concentration of one of the other components in the minimal medium. Varying the concentration of ammonium nitrate, potassium phosphate, or magnesium sulfate had no effect on the growth rate of the organism. Apparently these salts were not limiting the growth rate of the bacteria, nor were they toxic materials in the growth medium. The pH in the media increased from 7.2 to 7.8 by the end of the 72-hour

Figure 2. Effect of substrate (AIB) concentration (0.001 to 0.05%) on the growth of the organism. Growth was determined by OD at 600 nm after 72 hours of incubation.

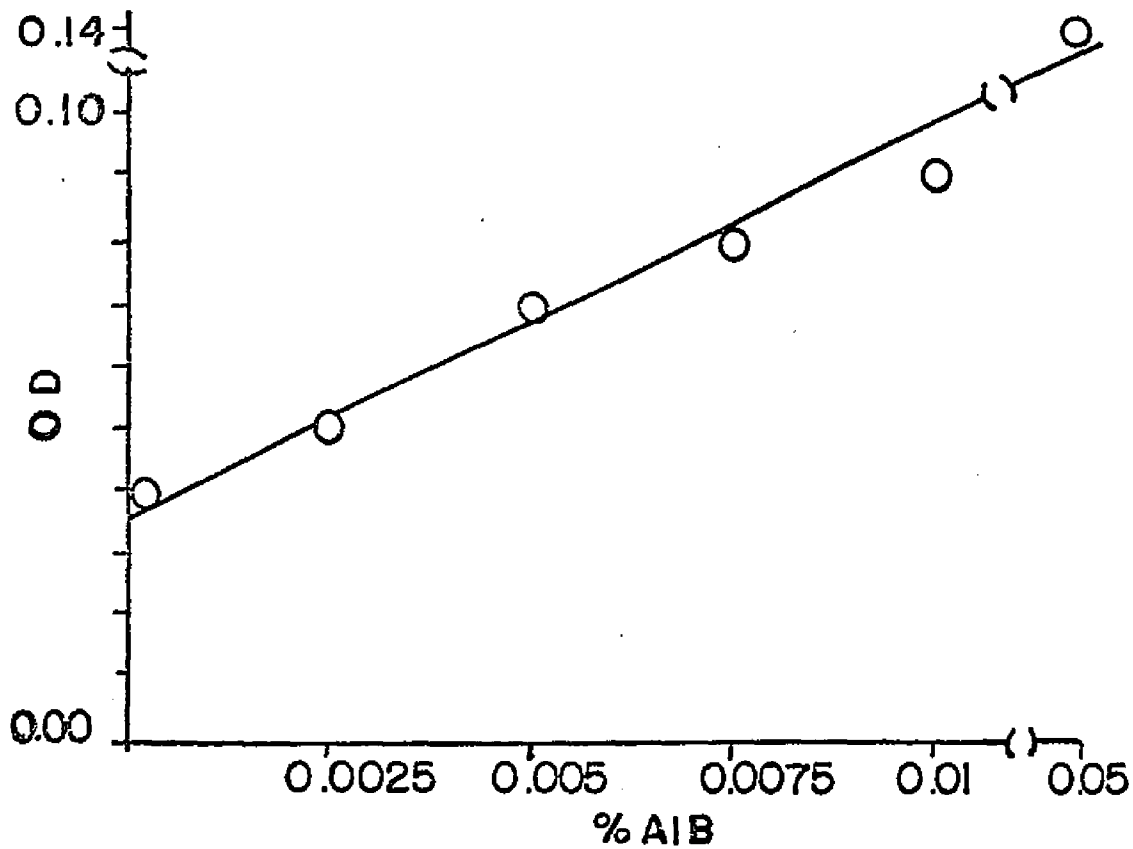
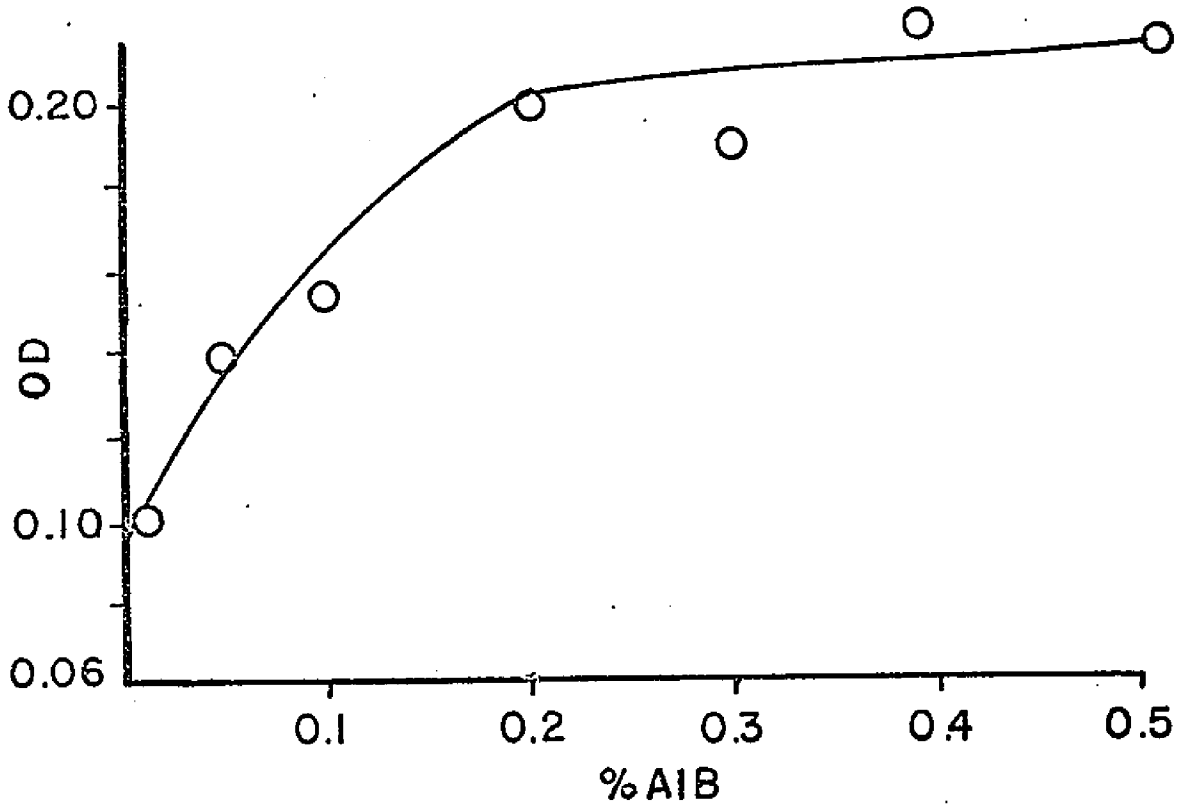

0.14
0.10
0.00
O.D
0.0025
0.005
0.0075
0.01
0.05
% AIB

Figure 3. Effect of substrate (AIB) concentration (0.01 to 0.5%) on the growth of the organism. Growth was determined by OD at 600 nm after 72 hours of incubation.


0.20
0.10
0.06
OD
0.1
0.2
0.3
0.4
0.5
% AIB

incubation period. This slight change in pH was not considered sufficient to cause the logarithmic death phase.

Acetone was the first product in the catabolism of AIB and the ability of the ketone to support growth of the bacterium was tested. The organism was inoculated into flasks containing different acetone concentrations in Stanier's salt mixture. At the end of 72 hours the optical densities of the cultures were determined. The results of this experiment are shown in Table 1. The organism grew best at an acetone concentration of 0.1% with an indication of toxicity at 0.2 and 0.3%. Higher concentrations of acetone were quite toxic as indicated by the low levels of growth of the organism.

Acetone also inhibited growth of the bacterium in nutrient broth as shown in Table 2.

The inhibition by acetone seemed to justify the determination of the ketone accumulation in AIB medium. A two-liter flask containing one liter of AIB minimal medium was inoculated and incubated at room temperature on rotatory shakers. Viable counts and acetone release into the medium were determined at different time intervals during a period of 72 hours. The results of this experiment are shown in Figure 4. Acetone was

Table 1

Growth of the organism with acetone as sole carbon source.

Flask no.	% acetone	OD[1]
1	0.1	0.21
2	0.2	0.11
3	0.3	0.14
4	0.4	0.07
5	0.5	0.085
6	0.6	0.06

[1]The OD was determined after 72 hours.

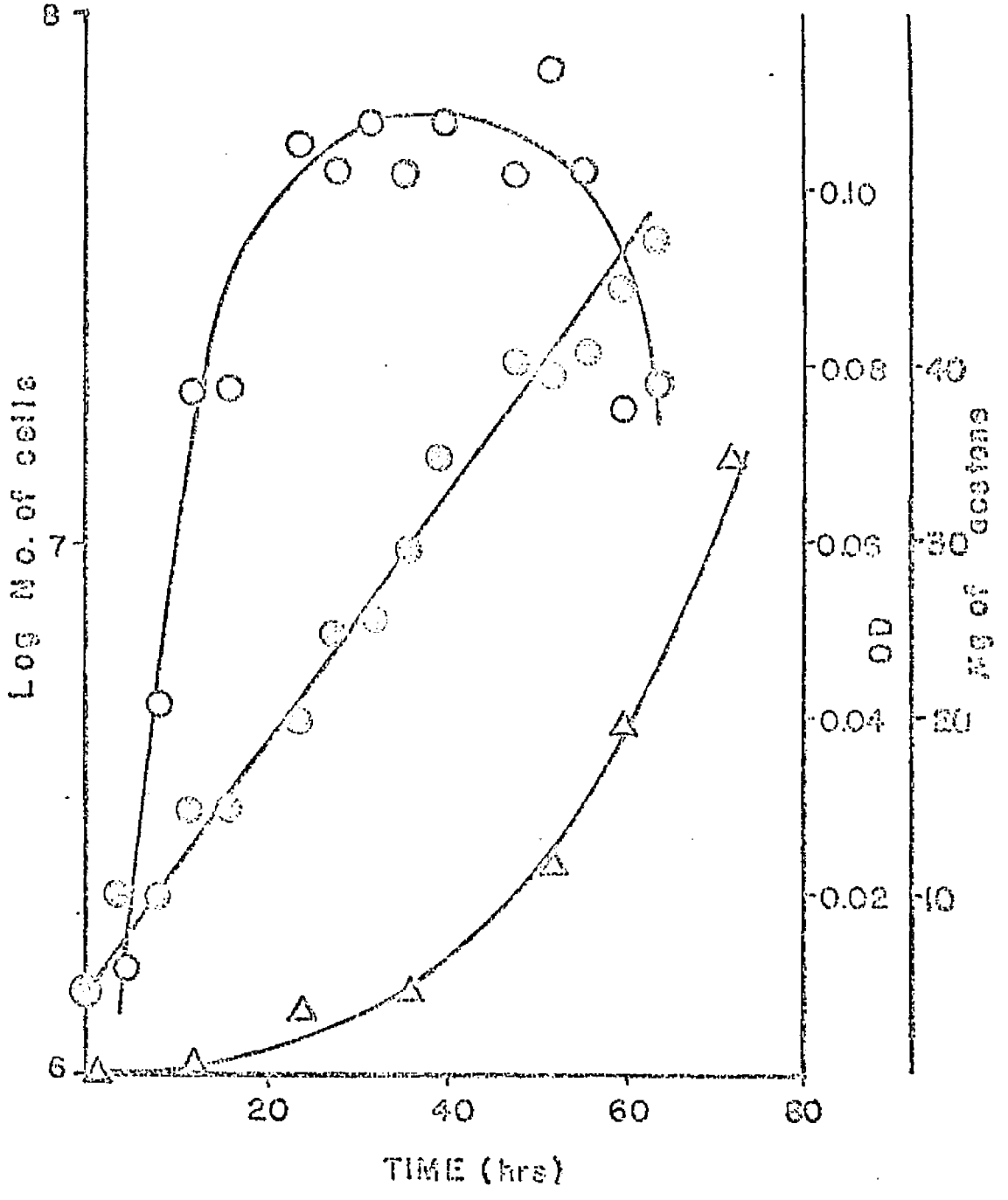
Table 2

Growth of the organism in nutrient broth plus acetone

Flask no.	% acetone	OD[1]
1	0.00	0.585
2	0.05	0.469
3	0.10	0.444
4	0.50	0.398
5	1.00	0.357
6	2.00	0.187

[1]The OD was determined after 18 hours.

Figure 4. Growth curve of the organism in AIB minimal medium. The bacteria were grown in 2-liter flasks containing 1 liter of AIB minimal medium. The viable counts (○), OD (●) and acetone accumulation (Δ) in the medium were determined.


8
7
6
Log No. of cells
0.10
0.08
0.06
0.04
0.02
OD
40
30
20
10
No. of colonies
20
40
60
80
TIME (hrs)

continuously released into the media, reaching a maximum of 35 µg/ml at 72 hours. Because the organism was capable of utilizing acetone as sole carbon source up to 3000 µg/ml, it seemed unlikely that the level of acetone accumulation shown in this experiment could account for the log death phase of the growth curve.

Because it was not apparent why the organism grew poorly on AIB medium, an attempt was made to increase the cell yield by adding catalytic amounts of supplements considered necessary in the catabolism of AIB. The decarboxylation-dependent transaminase, which catalyzed the initial reaction of AIB degradation, required PLP and pyruvate for catalytic activity. Therefore, the effect of pyridoxine and pyruvate supplements on the growth rate was determined. Supplementing the medium with 10, 40, 60, 80 and 100 ug/ml of pyridoxine had no effect on the growth rate of the bacteria. Similar negative results were obtained when pyruvate was used as a supplement. Acetol was suspected as being one of the intermediates in the catabolism of AIB. Since thiamine could be involved in the breakdown of this compound, the effect of the vitamin on the growth rate of the organism was tested. Supplementing the medium with 10, 20, 30, 40, 50, 60 and 100 ug/ml of the vitamin did not increase the cell yield.

Induction

In an effort to increase growth and still maintain the decarboxylation-dependent transaminase levels of the organism, the effect of a combination of acetone and AIB as carbon sources on the decarboxylation-dependent transaminase production was determined.

Cells were grown in two-liter flasks containing one liter of AIB medium or AIB medium plus 0.1% acetone. The flasks were incubated for 72 hours at room temperature on rotary shakers. The cells were then harvested, washed in buffer A, and sonified. The crude extracts were assayed for decarboxylation-dependent transaminase activity. The results of this experiment are shown in Table 3. Although acetone increased the cell yield slightly, the specific activity of decarboxylation-dependent transaminase in the extract was decreased by 70%.

The formation of decarboxylation-dependent transaminase using AIB as the sole nitrogen source was also investigated. The organism was grown on glucose, glycerol or acetate as carbon sources with AIB as the nitrogen source. The results of this experiment are shown on Table 4. Cells grown on glucose or acetate had no decarboxylation-dependent transaminase activity, while cells grown on glycerol had only 40% the activity of cells grown on AIB.

Table 3

Effect of combining acetone and AIB as carbon sources on decarboxylation-dependent transaminase production

Carbon source	OD[1]	Specific activity[2]
AIB (0.2%)	0.220	0.0200
AIB (0.2%) + acetone (0.1%)	0.367	0.0063

[1]The OD was determined after 72 hours.

[2]The reaction mixture contained 6.6 µmoles/ml of AIB, 6.6 µmoles/ml of pyruvate, 0.166 µmoles/ml of PLP, 13 µmoles/ml of phosphate buffer (pH 7.8) and 0.66 µmoles/ml EDTA (pH 7.0) and cellular extract.

Table 4

Formation of decarboxylation-dependent transaminase with AIB and/or NH_4NO_3 as nitrogen source.

Carbon source	Nitrogen source[1]	Specific activity[2]
AIB (0.2%)	AIB, NH_4NO_3	0.05
Glucose (0.5%)	AIB	0.00
Glycerol (1%)	AIB	0.02
Acetate (0.5%)	AIB	0.00
Alanine (0.2%)	NH_4NO_3	0.00

[1] NH_4NO_3 0.1%, AIB 0.2%.

[2] The reaction mixture was the same as described in Table 3.

The organism grew well on nutrient broth or in Stanier's salts plus 1% glycerol or glucose. An attempt was made to induce resting cells previously grown on the above substrates. Cells were grown on glycerol, glucose, or nutrient broth for 24 hours. The cells were harvested, washed three times in buffer A and suspended for 1 to 24 hours either in AIB medium or in 0.1% phosphate buffer (pH 7.2) plus 0.2% AIB. The cells were harvested again, washed in buffer A and sonified. The crude extracts were assayed for decarboxylation-dependent transaminase activity. The results of this experiment are shown in Table 5. No induction of decarboxylation-dependent transaminase on resting cells was observed in any case.

Purification of Decarboxylation-Dependent Transaminase

An attempt was made in this study to simplify the purification procedure of decarboxylation-dependent transaminase as outlined by Aaslestad (1). The general scheme as proposed by the above investigator is shown below:

1. Preparation of cellular extract.
2. High speed centrifugation.
3. First protamine sulfate precipitation.
4. Heat treatment.
5. First ammonium sulfate fractionation.
6. Second protamine sulfate precipitation.
7. Second ammonium sulfate fractionation.

Table 5

Formation of decarboxylation-dependent transaminase in resting cells

Substrate	Incubation Time (hrs)	Starvation Time (hrs)[1]	Induction Time (hrs)[2]	Specific Activity[3]
Glucose (0.5%)	48	0	1	0.00
Glycerol (1%)	24	0	1	0.00
Nutrient broth	24	0	1	0.0047
Nutrient broth	24	24	24	0.0105
Nutrient broth	96	48	24	0.00435
Nutrient broth	24	0	0	0.002526
Nutrient broth	48	0	1	0.00369

[1]Starvation was performed in 0.1% phosphate buffer pH 7.2.

[2]Induction was performed in AIB minimal media or in 0.1% phosphate buffer pH 7.2 plus 0.2% AIB.

[3]The reaction mixture was the same as described in Table 3.

By this procedure the decarboxylation-dependent transaminase could be purified ten-fold. It was found that steps 2 and 6 were the only steps which could be by-passed and still maintain the same degree of purification. When the second protamine sulfate treatment was not performed the enzyme was found in the 20-60% ammonium sulfate fraction.

Bouis (6) reported that acetone fractionation of the enzyme as described in Materials and Methods, after the second ammonium sulfate precipitation, accomplished a further five-fold purification of the decarboxylation-dependent transaminase. The enzyme was found in the precipitate obtained from the ratio of 1:1 (v/v) acetone to enzyme solution. However, in the present work his procedure only resulted in a 2.5-fold purification increase and the enzyme was found in the v/v 0.75 fraction. An attempt was made to by-pass the second ammonium sulfate fractionation (step 5) and the acetone precipitation by passing the first ammonium sulfate precipitate (0-45) through a 2.5 x 120 cm G-200 Sephadex column previously equilibrated with buffer A. The effect of direct acetone precipitation of the 0-45% ammonium sulfate fraction was also investigated.

Finally the enzyme was precipitated by a second ammonium sulfate fractionation (20-60%) followed by acetone fractionation (v/v = 0.75) and subsequently

passed through a Sephadex column. A 65-fold purification was obtained by this procedure.

Table 6 summarizes the results of these experiments.

Characterization of the Decarboxylation-Dependent Transaminase

Enzyme resolution. The enzymatic mechanism proposed by Aaslestad et al. (2) for the reaction catalyzes by the decarboxylation-dependent transaminase is shown below:

$$\text{AIB} + \text{PLP} \longrightarrow \text{acetone} + CO_2 + \text{PMP}$$
$$\text{PMP} + \text{pyruvate} \longrightarrow \text{PLP} + \text{alanine}$$

The same investigators reported that PLP could easily be removed by dialyzing the crude extract against Tris buffer. The activity of the apoenzyme was restored upon addition of PLP. PMP was found to be 2/3 as effective as PLP for activation of the crude extracts.

In order to further characterize the enzyme, dialysis experiments were performed to determine the binding capacities of the aldehyde and amino forms of the coenzyme to the apoenzyme. Three ml of an enzyme (18 mg/ml) preparation having a specific activity of 0.1452 was divided equally among three test tubes containing PLP (0.5 μmoles), PMP (0.5 μmoles) or PLP (0.5 μmoles) plus AIB (20 μmoles). The mixtures were brought to 3.6 ml with buffer A and they were individually

Table 6

Specific activities of fractions during purification of the decarboxylation-dependent transaminase

Step	A[1]	B	C	D
Cellular extract	0.03[2]	0.03	0.034	0.022
1st ammonium sulfate fractionation (0-.45)	0.053	0.114	0.228	0.096
2nd ammonium sulfate fractionation (.20-.6)	0.196	NP[3]	NP	0.15
Acetone v/v (.75) fractionation	0.52	NP	0.774	0.363
Sephadex G-200	NP	0.657	NP	1.44
Fold purified	17.3	21.9	22.9	65.5

[1]A, B, C and D represent different crude cellular extract preparations. The reaction mixture was the same as described in Table 3.

[2]Numbers represent specific activity.

[3]NP - not performed.

dialyzed against 0.2 M KCl at 4 C. At the end of four hours the samples were assayed in the presence and absence of PLP and the percent resolution was calculated. The data from this experiment are shown in Table 7. The results indicated that PLP is more tightly bound to the enzyme than PMP and that the substrate, AIB, aided the resolution of the aldehyde form of the enzyme. In order to confirm these results samples A and C were dialyzed again in the presence of AIB and PLP, respectively, and assayed in the presence and absence of PLP. The result of this experiment is also shown in Table 7. The resolution of sample A increased to 60% in the presence of substrate while the resolution of sample C decreased to 11% in the presence of PLP.

To determine to what extent resolution of the enzyme was dependent on extended dialysis in phosphate buffer the following experiment was designed. An enzyme preparation having a specific activity of 0.174 was dialyzed for two hours against a solution containing either KCl (0.2 M), PLP (50 μg/ml) and AIB (10 μg/ml) or KCl (0.2 M) and PLP (50 μg/ml). The enzyme preparations were then dialyzed against buffer A for 6 and 24 hours. The results of this experiment are shown in Table 8. Table 9 shows the effect of pyruvate and AIB on the resolution of PLP and PMP. PLP bound more

Table 7

Resolution of the decarboxylation-dependent transaminase

Sample	System[1]	Specific activity[3] -PLP	+PLP	% resolution
A	Enz + PLP	0.1278	0.1428	10
B	Enz + PMP	0.0981	0.1278	23
C	Enz + PLP + AIB	0.04425	0.1158	60
D[2]	A + AIB	0.0489	0.1215	60
E[2]	C + PLP	0.0906	0.1035	11

[1]All systems were dialyzed against 0.2 M KCl for 4 hours after addition of PLP, PMP or AIB.

[2]Samples D and E consisted of addition of PLP or AIB to samples A and C after dialysis and then dialyzed a second time.

[3]The reaction mixture was the same as described in Table 3.

Table 8

Effect of prolonged dialysis on decarboxylation-dependent transaminase resolution

Sample	System[1]	Dialysis time (hrs)[2]	Specific activity[3] -PLP	+PLP	% resolution
A	Enz + PLP + AIB	6	0.1038	0.1518	31.7
B	Enz + PLP + AIB	24	0.02964	0.1398	81.0
C	Enz + PLP	6	0.1314	0.1587	17.2
D	Enz + PLP	24	0.0609	0.1035	41.0
E	Enz + KCl	6	0.0713	0.1740	59.5

[1]Systems were obtained by dialyzing the enzyme for 2 hours against solutions containing either KCl (0.2 M), PLP (50 µg/ml) and AIB (10 µg/ml) or KCl (0.2 M) and PLP (50 µg/ml). Sample E was dialyzed for 2 hours against 0.2 M KCl.

[2]Dialyzed against buffer A.

[3]The reaction mixture was the same as described in Table 3.

Table 9

Effect of pyruvate and AIB on the resolution of decarboxylation-dependent transaminase.

Sample	System[1]	% resolved[2] before dialysis	% resolved[2] after dialysis
A	Enz + PLP	60	11
B	Enz + PLP + Py	60	0
C	Enz + AIB + PMP	97	82
D	Enz + AIB + PMP[3]	97	61

[1]The conditions were the same as described in Table 7, except that dialysis was performed against buffer A.

[2]The % resolution was determined before and after experimentation.

[3]Sample D was dialyzed against buffer A plus 0.2% AIB.

firmly in the presence of pyruvate and it was apparent that excess AIB slightly increased PMP binding.

Mechanism of enzyme action. The ease with which the holoenzyme resolved into coenzyme and apoenzyme in the presence of AIB made it appear feasible that stoichiometric amounts of PLP or PL could replace the amino acceptor pyruvate. If the enzymatic reaction mechanism was functioning as previously proposed once a molecule of AIB reacted with the enzyme, the resulting PMP would leave the active site which could then be reoccupied by another molecule of PLP. The results of this experiment are shown in Table 10. The results showed that stoichiometric amounts of PLP or PL would not replace pyruvate. The results in Table 11 demonstrated that PLP cannot replace the keto acid even in the presence of catalytic amounts of pyruvate.

The requirement for pyruvate for the enzyme led to the consideration that PLP was not a coenzyme but rather a structural component involved in quaternary structure of the enzyme. To test this hypothesis the following experiment was designed. An enzyme preparation having a specific activity of 1.44 was resolved to 63% and 400-ul aliquots were placed on 5-20% sucrose gradients. One of the gradients was prepared

Table 10

Inability of PLP to replace pyruvate in the decarboxylation-dependent transaminase

Sample	% resolved	System[1]	Reaction time (min)	Activity[2] CO_2	Activity[2] Acetone
A	23	AIB + PLP	10	ND[3]	0.28
B	23	AIB + PLP + Py	10	ND	1.04
C	60	AIB + PLP	300	0	0.00
D	60	AIB + PLP + Py	300	77.7	4.95
E	63	AIB + PLP + Py	60	24	1.16
F	63	AIB + PLP	60	0	0.00
G	63	AIB + PL	60	0	0.00
H	78	AIB + Py	60	ND	0.103

[1]Samples A, C and F contained stoichiometric amounts of PLP (10 μmoles/ml). Samples B, D and E contained catalytic amounts of PLP (0.166 μmoles/ml). Sample G contained 10 μmoles/ml of PL. AIB and Py were used in stoichiometric amounts (6.6 μmoles/ml). All samples contained 13 μmoles/ml of phosphate buffer (pH 7.8) and 0.66 μmoles/ml of EDTA (pH 7.2).

[2]Activities of samples A, B, C and D were expressed in ul of CO_2/mg of protein or μmoles of acetone/mg of protein. Activities of samples E, F, G and H were expressed in μl of CO_2 or μmoles of acetone produced. These samples contained the same amount of protein.

[3]ND - not determined.

Table 11

Inability of PLP to replace pyruvate in the decarboxylation-dependent transaminase in the presence of catalytic amounts of the keto acid

% resolved	System[1]	Reaction time (min)	Activity[2] CO_2	Activity[2] acetone
63	1	60	1.2	0.076
63	2	60	19.3	0.600

[1]System 1 Py = 0.16 µmoles/ml
PLP = 4.3 µmoles/ml
AIB = 6.5 µmoles/ml

System 2 Py = 6.8 µmoles/ml
PLP = 0.175 µmoles/ml
AIB = 6.5 µmoles/ml

Both systems contained 13 µmoles/ml of phosphate buffer (pH 7.8), 0.66 µmoles/ml of EDTA (pH 7.0) and the same amount of enzyme.

[2]Activity was expressed as ul CO_2 or µmoles of acetone produced.

in the presence of excess PLP. The other gradient contained no PLP. Both gradients were centrifuged using a SW 39 rotor in a Spinco Model L ultracentrifuge for 18 hours at 37,000 rpm. At the end of the run 20-drop fractions were collected by puncturing the bottom of the plastic tubes containing the gradient and the fractions were assayed for decarboxylation-dependent transaminase activity. The results shown in Figure 5 demonstrated that identical activity peaks existed under both experimental conditions.

To further elucidate the role of PLP as a functional coenzyme 65-fold purified enzyme was first resolved as previously described and then reduced with $NaBH_4$ in the presence and absence of PLP. The resolved enzyme was distributed equally into two test tubes. One of the test tubes contained 0.05 µmoles of PLP. The volume was adjusted with 0.2 M KCl, and both enzyme preparations were dialyzed for four hours against 0.2 M KCl. The samples were then dialyzed for five minutes against 0.005 M $NaBH_4$ to reduce the cofactor to the apoenzyme. The reduced preparations were then dialyzed against a solution containing AIB (0.2%) and KCl (0.2 M) for two hours to remove excess PLP. Both samples were assayed in the presence and absence of PLP. An outline of the procedure and the results of the experiment are shown in Figure 6. The

Figure 5. Sucrose density gradient centrifugation of apoenzyme in the presence (●) and absence (○) of PLP. Twenty drop fractions were collected and assayed for decarboxylation-dependent transaminase activity. Activity was expressed in OD units measured at a wave length of 420 nm in a DB Beckman spectrophotometer. The reaction mixture was the same as described in Table 3.

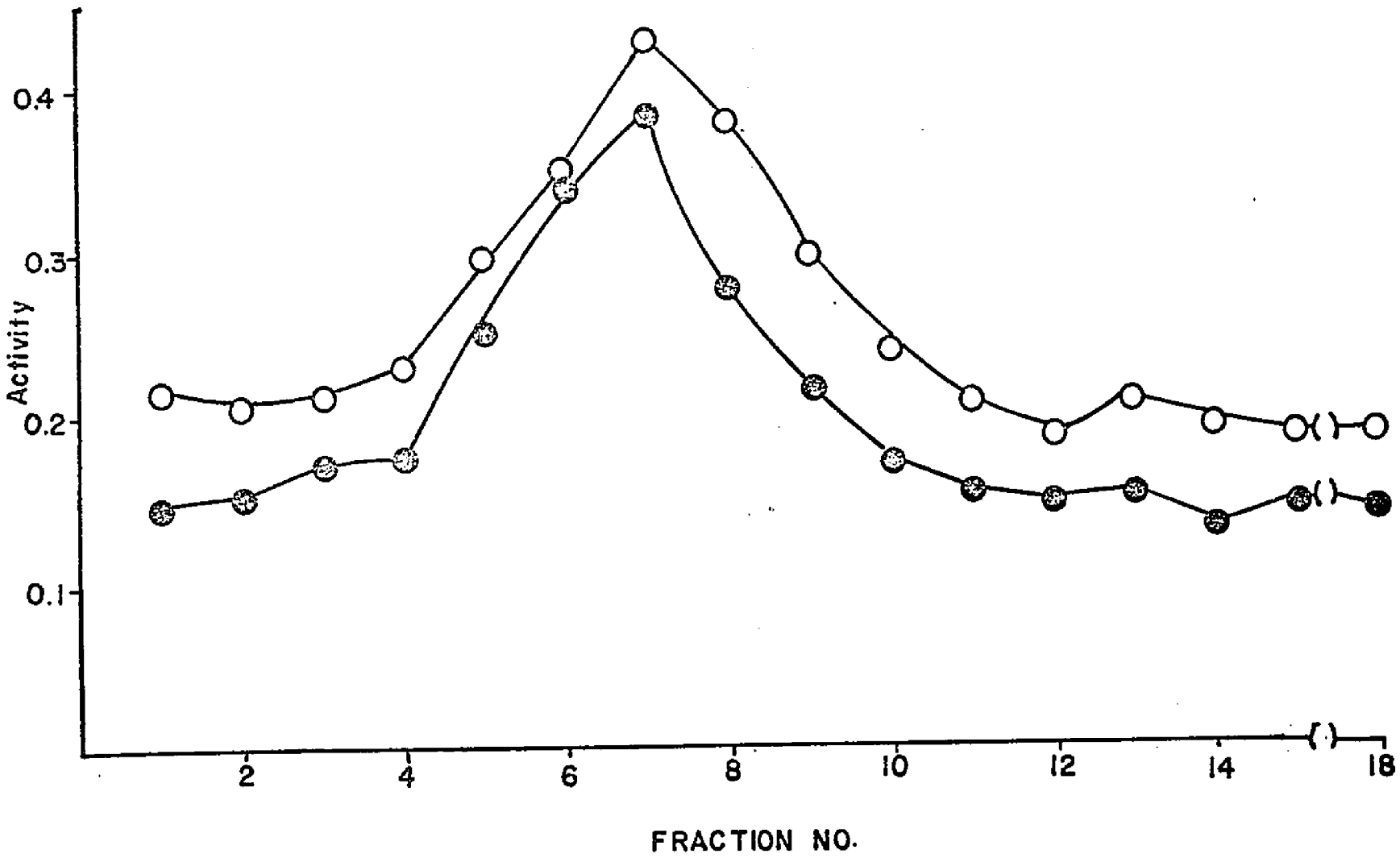

0.4
0.3
0.2
0.1
Activity
2
4
6
8
10
12
14
18
FRACTION NO.

Figure 6. Sodium borohydride reduction of holo- (**A**) and apoenzyme (**B**). Activity was assayed in the presence and absence of PLP and was expressed in umoles of acetone produced. The reaction mixture was the same as described in Table 3.

RESOLVED ENZYME

Sample A
0.2 ml of PLP (2.5 μmoles/ml) was added

Sample B
0.2 ml of KCl (0.2 M) was added

Procedure performed on both samples:

1) Dialyzed against 0.2 M KCl for 4 hours.
2) Dialyzed against 0.005 M $NaBH_4$ for 5 min.
3) Dialyzed against 0.2 M KCl plus 0.2% AIB for 2 hours.

Activity of Sample A	
+PLP	-PLP
0.086	0.00

Activity of Sample B	
+PLP	-PLP
0.93	0.00

enzyme which was reduced in the presence of PLP had only one-tenth of the activity of the enzyme which was reduced in the absence of PLP.

The 65-fold purified enzyme was analyzed by disc-gel electrophoresis. Eight bands appeared on the gel, two of which were markedly dense. The presence of the two dense bands suggested that two different proteins might be involved in the decarboxylation-dependent transamination reaction. In model systems isopropylamine was one of the products in the decarboxylation-dependent transamination reaction. If two proteins were involved in the reaction the following mechanism was visualized:

A) AIB $\longrightarrow$ ISPA + CO_2

B) ISPA + PLP $\longrightarrow$ acetone + PMP
PMP + pyruvate $\longrightarrow$ PLP + alanine

ISPA and acetone were tested for their capacity to inhibit reaction A.

The effect of acetone on the decarboxylation-dependent transaminase was determined. The enzyme was assayed for activity by measuring CO_2 production in the presence of different concentrations of acetone. The results are presented in Table 12 and provided no evidence of acetone inhibition.

The effect of ISPA on the decarboxylation-dependent transaminase was determined. The enzyme was

Table 12

Activity of decarboxylation-dependent transaminase in the presence of varied amounts of acetone

Sample	Acetone (μmoles/ml)	Activity[1]
A	1	10.7
B	4	9.9
C	7	7.2
D	10	7.2
E	20	9.6
F	0	7.2

[1]Activity was expressed in μl of CO_2 released. All samples contained the same amount of protein. The reaction mixture was the same as described in Table 3.

assayed for decarboxylation-dependent transaminase activity in the presence of different concentrations of ISPA. The results of this experiment are shown in Table 13. ISPA did not inhibit the decarboxylation-dependent transaminase. The enzyme preparation was also assayed for ISPA-pyruvate transaminase activity. The results of this investigation are presented in Table 14. No evidence for such a transamination reaction was obtained.

The two heavy protein bands in the gel may indicate that two enzymes were involved each catalyzing separate reactions in the mechanism proposed by Aaslestad et al. (2). The proposed mechanism, whether one or two enzymes were involved, required a PMP intermediate and PMP should replace PLP in the reaction. The enzyme was resolved and assayed in the presence of PMP or PLP, and the results shown in Table 15 indicated that the PMP intermediate was indeed involved. One can also conclude from these results that if the enzyme preparation contained a PMP-pyruvate transaminase the enzyme would not require PLP for catalytic activity.

The enzyme preparation was assayed for PMP-pyruvate transaminase activity. The addition of catalytic amounts of PLP to the reaction mixture was not considered necessary because of the evidence obtained

Table 13

Activity of decarboxylation-dependent transaminase in the presence of varied amounts of ISPA

Samples	ISPA (μmoles/ml)	Activity[1] CO_2	Activity[1] Acetone
A	0	19.8	0.611
B	1	24.2	0.715
C	4	19.8	0.705
D	6	ND[2]	0.637
E	10	19.8	0.741

[1]Activity was expressed in μl of CO_2 or μmoles of acetone produced. All samples contained the same amounts of protein. The reaction mixture was the same as described in Table 3.

[2]ND - not determined.

Table 14

Inability of the decarboxylation-dependent transaminase to transaminate between ISPA and pyruvate

Sample[1]	System	Activity[2]
A	ISPA + Py + PLP	0.00
B	ISPA + Py	0.00

[1]Sample A contained: ISPA = 16 µmoles/ml
Py = 6.6 µmoles/ml
PLP = 0.166 µmoles/ml

Sample B contained the same concentration of ISPA and Py as sample A. Both samples contained 10 µmoles/ml of phosphate buffer pH 7.8.

[2]The reaction was carried out in the presence of active decarboxylation-dependent transaminase in a total volume of 1 ml for 60 min, and the activity was determined by the appearance of acetone as described in Materials and Methods.

Table 15

Ability of PMP to replace PLP in the decarboxylation-dependent transaminase system.

Sample	System	Activity[1]	% activity of A
A	AIB + Py + PLP	0.466	100
B	AIB + Py + PMP	0.414	89
C	AIB + Py	0.103	22.9

[1]Activity was expressed in umoles of acetone produced. The reaction mixture was the same as described in Table 3, except that sample B contained PMP (0.166 µmoles/ml) and sample C did not contain PLP or PMP. All samples contained the same amount of protein.

previously. The reaction mixture containing stoichiometric amounts of PMP and pyruvate and resolved enzyme was incubated for one hour at 30 C.

Thin-layer chromatography was employed to detect any alanine produced. No evidence for PMP-pyruvate transaminase activity was obtained as indicated by the results shown in Figure 7.

Although the enzyme cannot catalyze transamination between PMP and pyruvate, this reaction should occur in a stoichiometric manner with enzyme. In order to determine if the stoichiometric transamination between PMP and pyruvate occurred the following experiment was designed. Two ml of resolved enzyme were divided equally between two test tubes. One contained PMP plus pyruvate while the other one contained only PMP. The reaction mixtures were incubated for 10 minutes at 30 C and subsequently dialyzed for 24 hours against buffer A. The outline and results of this experiment are shown in Figure 8. PLP was required for enzyme activity in both systems and it is apparent that in the absence of AIB transamination between PMP and pyruvate does not occur.

Enzyme kinetics. To elucidate further the mechanism of action of the decarboxylation-dependent transaminase, kinetic experiments were performed. Enzyme

Figure 7. Qualitative assay for transamination using thin-layer chromatography. The enzyme was incubated for 1 hour at 30 C with different reaction mixtures and a sample from the mixture was chromatographed. The components of the reaction vessel were the following:

Sample 1. AIB (6.6 µmoles/ml), Py (6.6 µmoles/ml) and PLP (0.166 µmoles/ml).
Sample 2. AIB (6.6 µmoles/ml) and Py (6.6 µmoles/ml).
Sample 3. Py (6.6 µmoles/ml) and PMP (7.5 µmoles/ml).
Sample 4. Py (6.6 µmoles/ml).
Sample 5. Same as no. 3 without enzyme.

In addition all reaction vessels contained 13 µmoles/ml of phosphate buffer pH 7.8 and 0.66 µmoles/ml of EDTA. Sample 6 was the alanine reference.

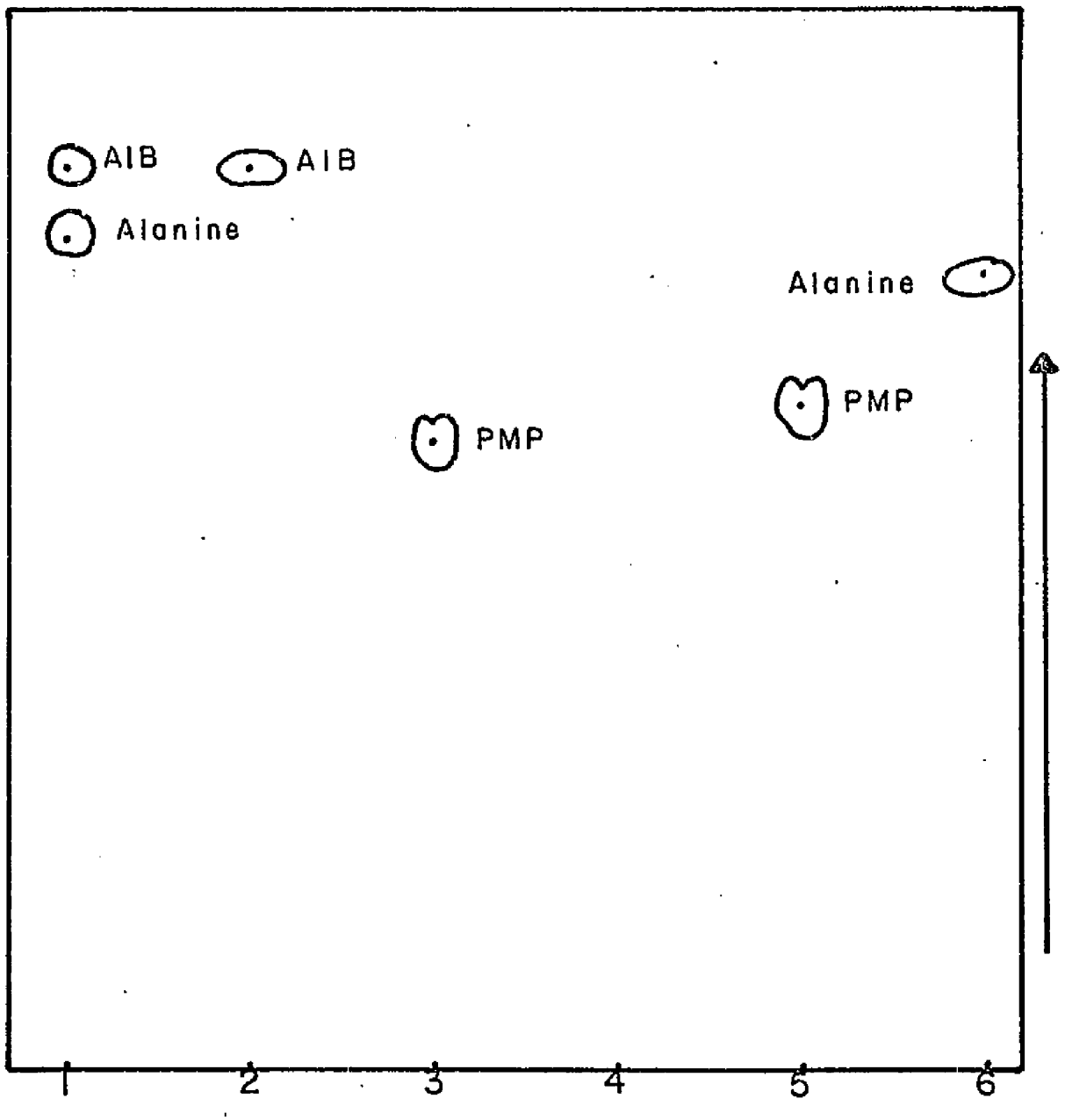

AIB
AIB
Alanine
Alanine
PMP
PMP
1
2
3
4
5
6

Figure 8. Inability of the enzyme to transaminate between PMP and pyruvate. Activity was expressed in μmoles of acetone produced. The reaction mixture was the same as described in Table 3. Both samples contained the same amount of protein.

RESOLVED ENZYME

Procedure performed on both samples:

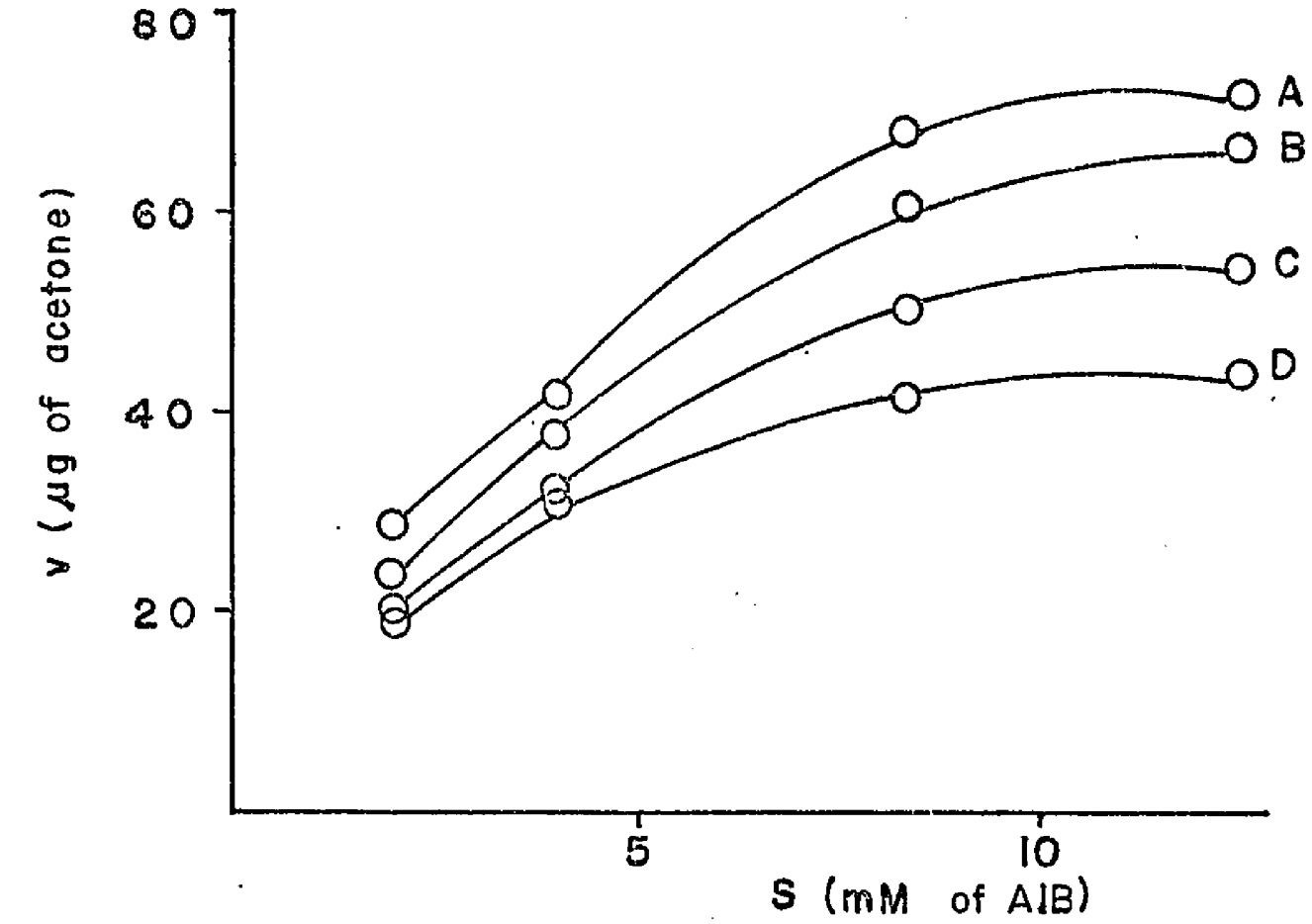
1) Incubation for 10 min. at 30 C.
2) Dialyzed for 24 hrs against buffer A.

Activity of Sample A	
+PLP	-PLP
2.7	0.604

Activity of Sample B	
+PLP	-PLP
2.3	0.45

purified 65-fold was assayed for acetone production maintaining the concentration of one substrate constant while varying the concentration of the other. The v vs S plots at varying AIB concentrations are presented in Figure 9a. Pyruvate concentration was maintained constant at 12, 8, 6 or 4 µmoles in a final volume of 1.2 ml. It was apparent that pyruvate was an inhibitor of the decarboxylation-dependent transamination reaction. Figure 9b shows the double reciprocal plots. The non-linearity of these plots was evident. Figure 10 shows a v vs S plot using 3.33 µmoles/ml of pyruvate and varying amounts of AIB. At these low concentrations of AIB the plots appeared sigmoidal. Figure 11 shows the double reciprocal plots and again the non-linearity of the curve was evident. Figure 12 represents plots of pyruvate versus activity employing constant concentrations of AIB (5 and 7 µmoles/ml). Inhibition of the enzyme by pyruvate was apparent at concentrations of 2 mM or higher. Figures 13 and 14 show the v vs S and double reciprocal plots at concentrations of pyruvate less than 2mM and at constant concentrations of AIB (8.8 or 17.6 µmoles/ml). Both v vs S plots, at 8.8 or 17.6 µmoles/ml of AIB, showed sigmoidal characteristics. The double reciprocal plots also gave non-linear curves.

Figure 9. Effect of pyruvate on the kinetics of the decarboxylation-dependent transaminase. a) Kinetic plot v vs S and b) double reciprocal plot. The velocity was measured in µg of acetone produced in 1 hour. The reaction mixture contained enzyme, 13 µmoles/ml of phosphate buffer pH 7.8, 0.66 µmoles/ml of EDTA and 0.166 umoles/ml of PLP. AIB concentration was varied from 2 to 12.5 mM. The constant amounts of pyruvate used to obtain the different curves were: A = 4 µmoles, B = 6 µmoles, C = 8 µmoles and D = 12 µmoles. Distilled water was added to make final volume of 1.2 ml.


80
60
40
20
v (μg of acetone)
A
B
C
D
5
10
S (mM of AIB)

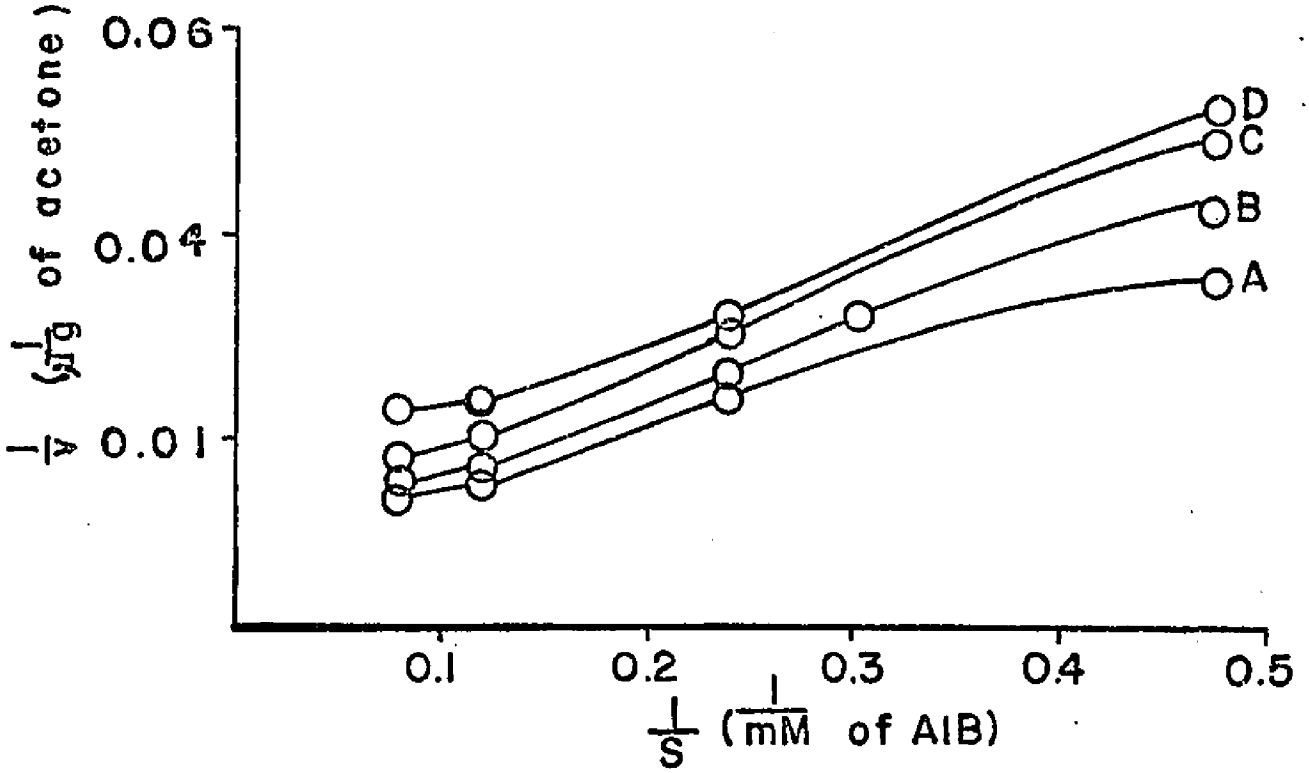

0.06
0.04
0.01
1/v (1/μg of acetone)
D
C
B
A
0.1
0.2
0.3
0.4
0.5
1/S (1/mM of AIB)

Figure 10. Kinetic plot of v vs S at constant pyruvate concentration (3.33 µmoles/ml). The velocity was measured in µg of acetone produced during 1 hour. The reaction mixture contained 13 µmoles/ml of phosphate buffer pH 7.8, 0.66 µmoles/ml of EDTA and 0.166 µmoles/ml of PLP. The concentration of AIB varied from 0.4 to 8 mM.

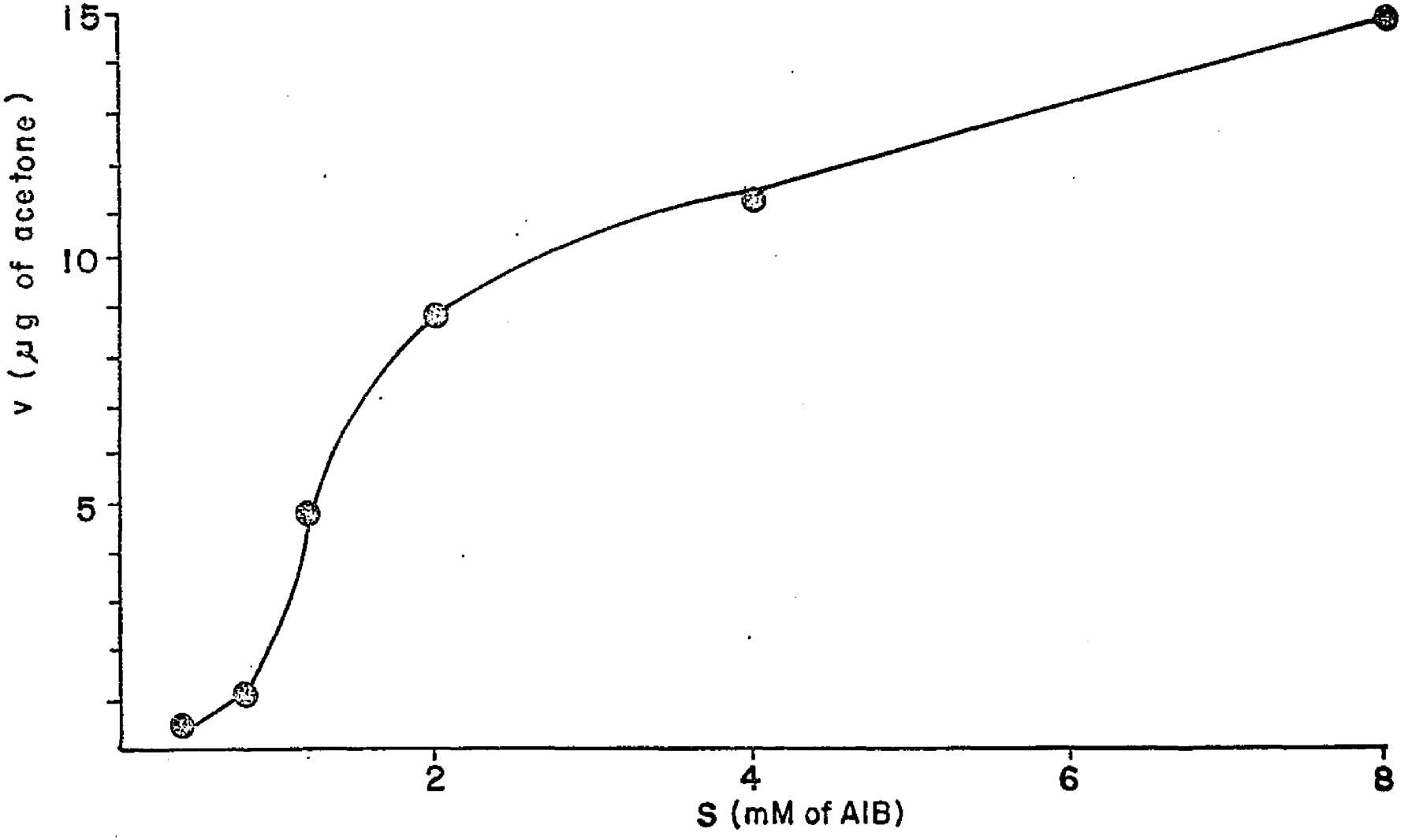

15
10
5
v (µg of acetone)
2
4
6
8
S (mM of AIB)

Figure 11. Double reciprocal plot at constant pyruvate concentration (3.33 µmoles/ml). The conditions were the same as described in Figure 10.

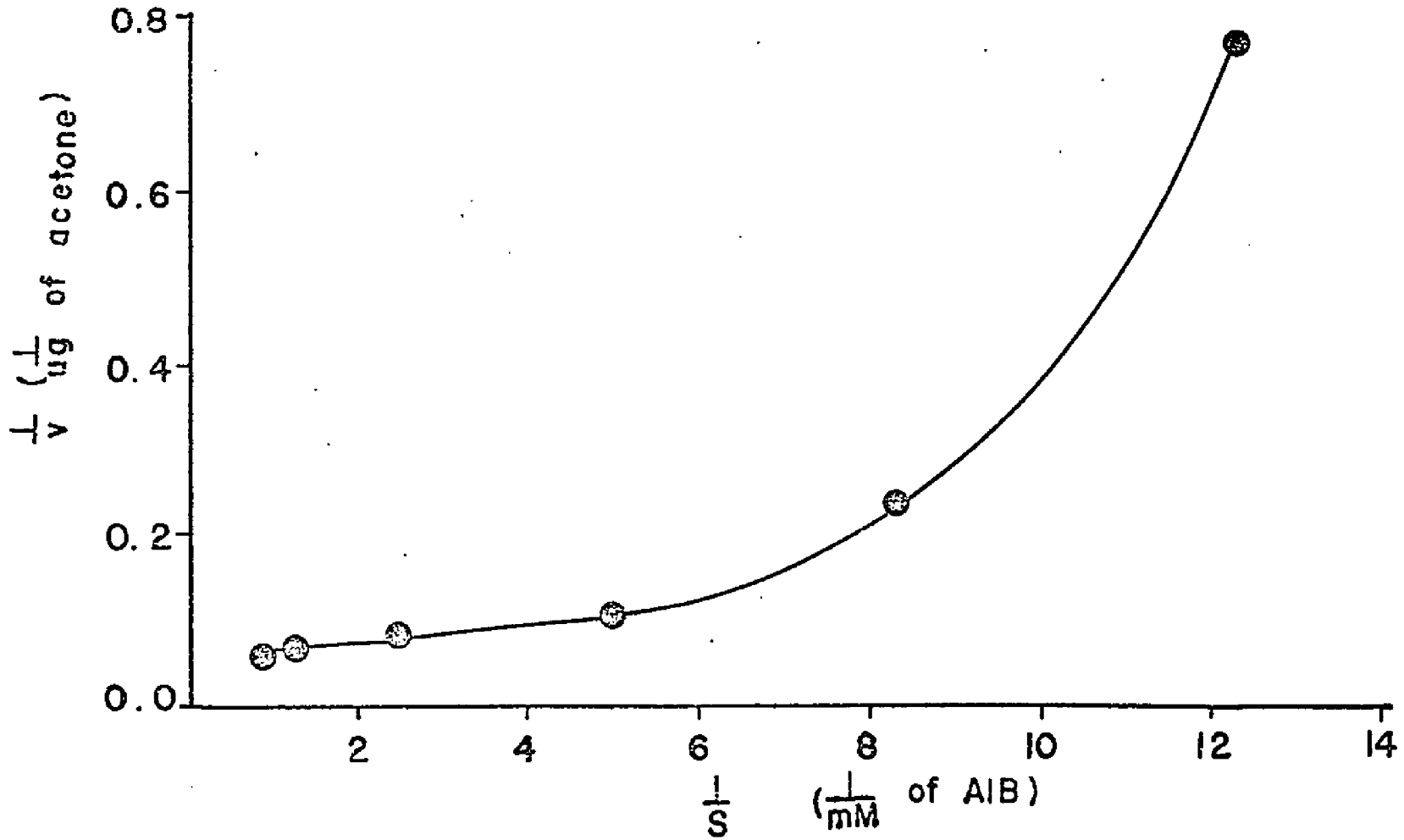

0.8
0.6
0.4
0.2
0.0
1/v (1/ug of acetone)
2
4
6
8
10
12
14
1/S (1/mM of AIB)

Figure 12. Kinetic plot of v vs S at constant AIB concentrations (Δ = 5 umoles/ml, O = 7 umoles/ml). Velocity was measured in μg of acetone produced during 1 hour. The conditions were the same as described in Figure 10 except that the pyruvate concentration was varied from 0.1 to 13.3 mM.

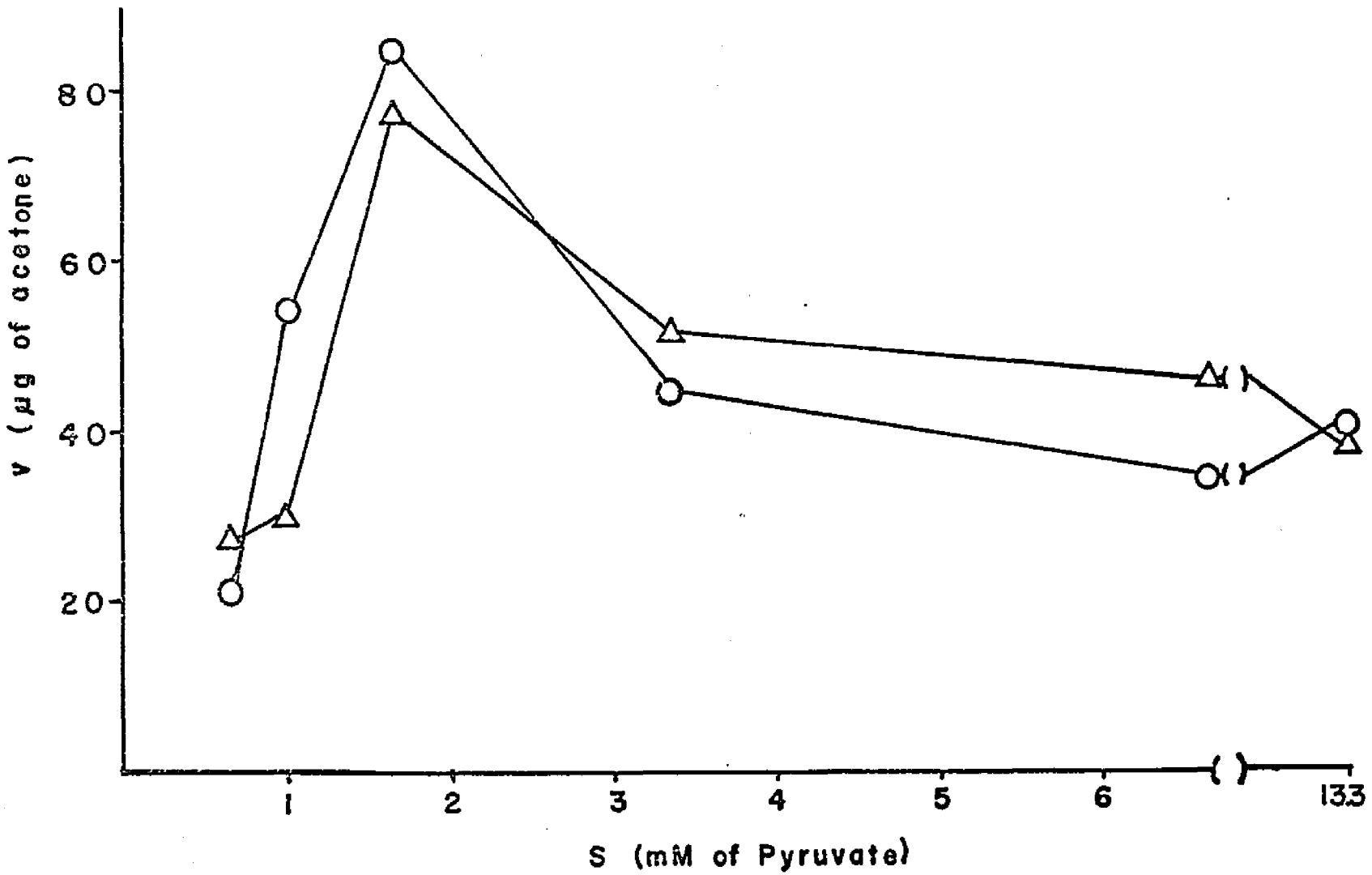

v (µg of acetone)
80
60
40
20
1
2
3
4
5
6
133
S (mM of Pyruvate)

Figure 13. a) Kinetic plot v vs S and b) double reciprocal plot at constant AIB concentration (8.82 umoles/ml). Velocity was measured in µg of acetone produced during 1 hour. The reaction mixture was the same as described in Figure 10 except that the pyruvate concentration was varied from 0.1 to 1.2 mM.

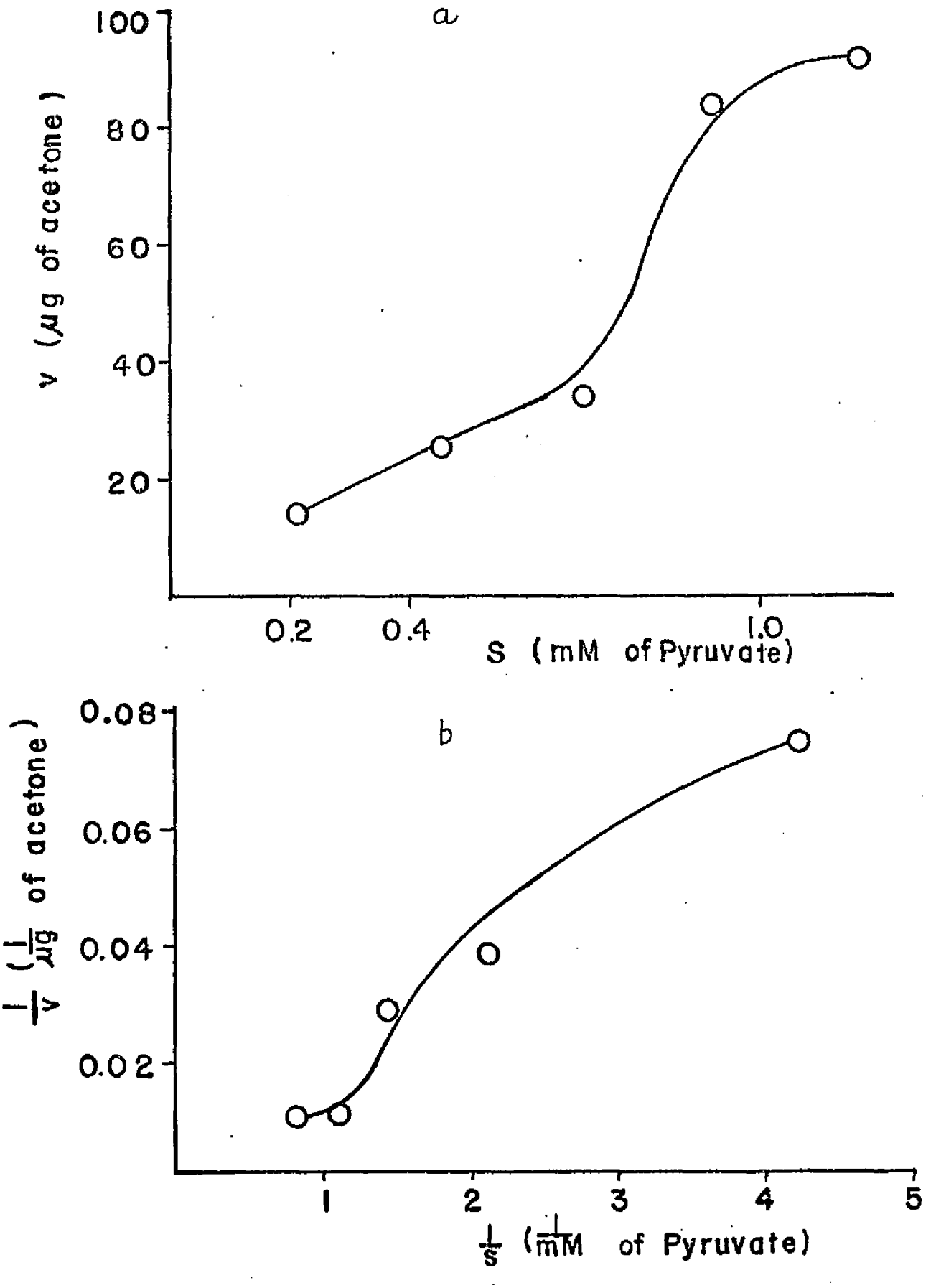
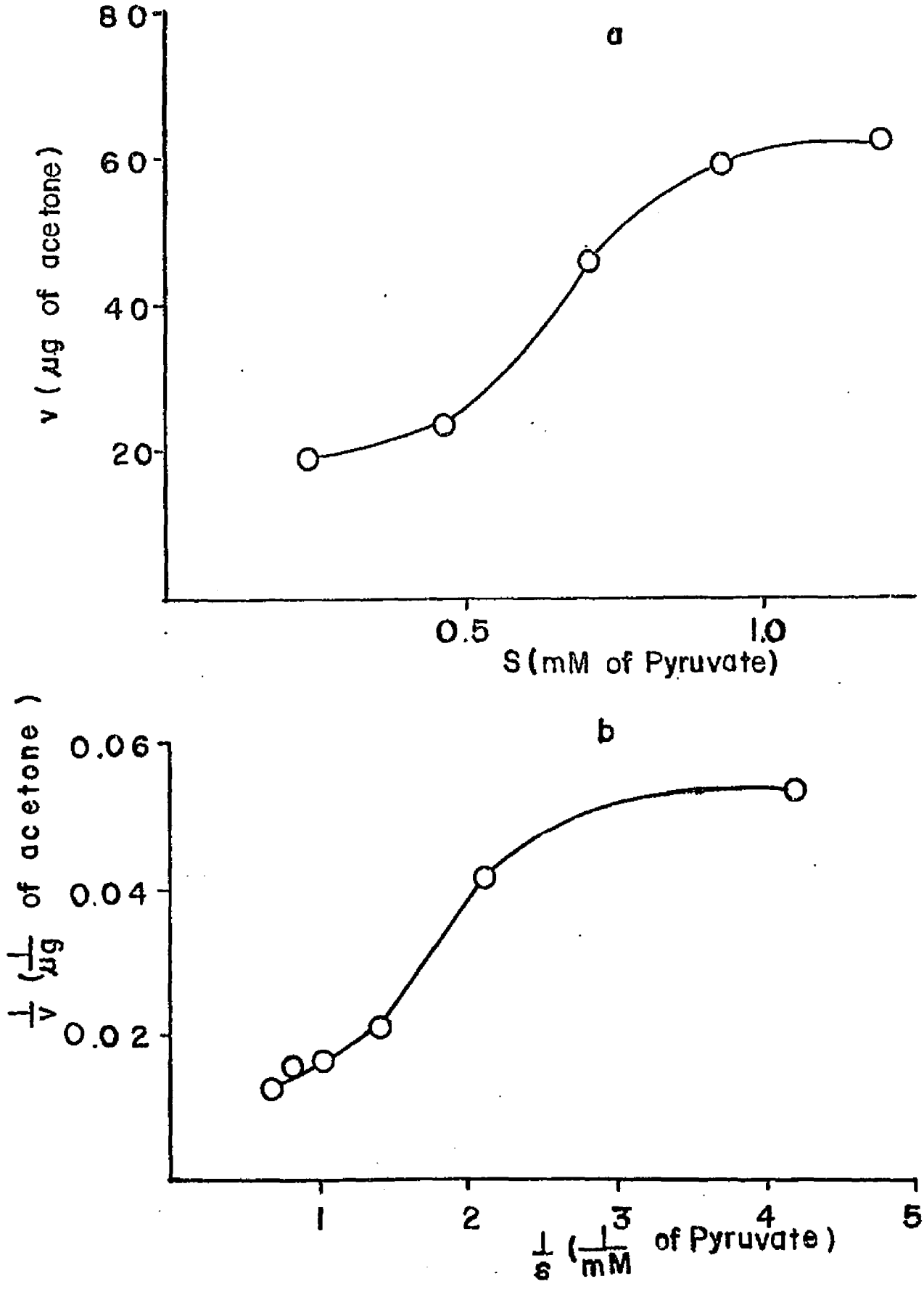
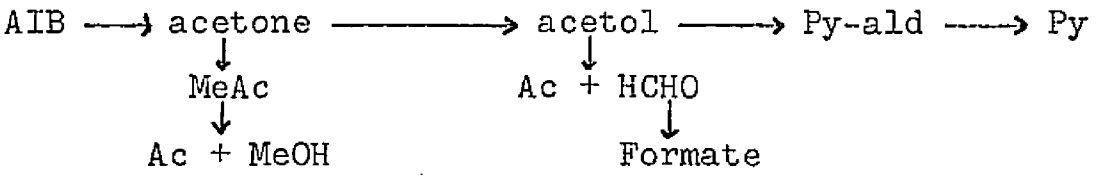

a
100
80
60
40
20
v (µg of acetone)
0.2
0.4
1.0
S (mM of Pyruvate)
b
0.08
0.06
0.04
0.02
1/v (1/µg of acetone)
1
2
3
4
5
1/S (1/mM of Pyruvate)

Figure 14. a) Kinetic plot v vs S and b) double reciprocal plot at constant AIB concentration (17.6 µmoles/ml). Velocity was measured in ug of acetone produced during 1 hour. The reaction mixture was the same as described in Figure 10 except that the pyruvate concentration was varied from 0.1 to 1.2 mM.


a
80
60
40
20
v (µg of acetone)
0.5
1.0
S (mM of Pyruvate)
b
0.06
0.04
0.02
1/v (1/µg of acetone)
1
2
3
4
5
1/s (1/mM of Pyruvate)

Catabolism of AIB

One of the first products in the catabolism of AIB was acetone. The organism when grown on AIB was essentially utilizing the ketone as its carbon source. The following pathways were proposed for the degradation of AIB or acetone:



The organism would not grow on pyruvic aldehyde, methyl acetate, methanol, formaldehyde or formate as the sole carbon source. However, the organism grew on AIB, acetone, acetol, pyruvate, acetate or glycerol as the sole carbon source. The cells were harvested, washed three times in buffer A and starved at room temperature by resuspending them in the same buffer for one or two hours. The starved cells were harvested again and resuspended in buffer A containing chloramphenicol (about 300 µg/mg dry weight of cells). A suspension of these cells containing 6 to 10 mg of cells dry weight was tested for the ability to oxidize the intermediates proposed in the pathway. The results are presented in Table 16. AIB was oxidized only by AIB-grown cells. Acetone was oxidized by AIB, acetone and acetol-grown cells. However, acetol-grown cells slowly oxidized

Table 16

Oxidation of proposed intermediates in the catabolism of AIB

Substrate	Oxygen uptake[1] of cells grown in					
	AIB	Acetone	Acetol	Acetate	Pyruvate	Glycerol
AIB	6.7	0.7	0.1	0.0	0.1	0.1
Acetone	5.2	4.6	2.7	0.0	0.1	0.1
Acetol	7.0	7.5	5.6	0.0	0.3	0.3
Py-ald	5.2	6.1	6.1	0.0	0.0	0.0
MeAc	5.3	3.2	1.7	0.0	0.5	0.5
MeOH	1.3	1.5	0.0	0.0	0.3	0.3
Acetate	3.7	3.1	1.8	3.6	2.1	2.1
Formate	0.0	1.0	0.0	0.0	0.8	0.8
Pyruvate	0.9	1.4	0.3	0.2	6.0	ND
Glycerol	ND[2]	ND	ND	ND	ND	6.0

[1]Oxygen uptake expressed in μmoles O_2 x 10/mg dry weight weight/100 min corrected for endogenous. Reaction vessel contained 4-5 μmoles/ml of substrate and buffer A (0.5 ml). The center well contained 0.2 ml of 20% KOH and the final volume of 3 ml was adjusted with distilled water.

[2]ND - not determined.

the ketone. Oxidation of acetol, pyruvic aldehyde and methyl acetate was observed with cells grown on AIB, acetone or acetol. Acetol-grown cells oxidized the ester to a lesser extent. Methanol was very slowly oxidized by cells grown on AIB or acetone. Oxidation of the alcohol was not detected with the rest of the cell preparations. Cells grown in all substrates tested oxidized acetate but not formate. Pyruvate was oxidized only by cells grown on pyruvate, even though acetone-grown cells showed some oxidation of the keto acid. It should be noted that acetate, glycerol and pyruvate-grown cells did not oxidize any of the intermediates and that acetate-grown cells did not oxidize pyruvate.

DISCUSSION

The organism used in this study exhibited a long generation time on AIB minimal media. The results obtained from the growth curves indicated the presence of some restricting factor in the medium which limited maximal growth of the organism. The limitation however, cannot be attributed to the depletion of carbon or nitrogen supply. Pyridoxine and thiamine did not increase the cell yield and yeast extract at low concentrations had the same effect as acetate. Yeast extract and acetate increased the cell yield at the expense of the decarboxylation-dependent transaminase formation. The levels of acetone accumulation in the medium (35 µg/ml) were not considered sufficient to inhibit growth, because the organism was capable of utilizing the ketone as sole carbon source up to a concentration of 0.3%. Since methyl acetate may be an intermediate in the catabolism of AIB and the organism cannot oxidize methanol, the effect of this alcohol on the growth of the organism should be investigated. Methanol may accumulate in sufficient quantities to inhibit cell growth.

The decarboxylation-dependent transaminase was only produced when the organism was growing on AIB as the sole carbon source. It was apparent that resting

cells cannot be induced to produce the enzyme under the conditions tested. The use of AIB as nitrogen source also proved ineffective for the induction of the enzyme.

The decarboxylation dependent transaminase was purified 65-fold from the crude cellular extract. The second ammonium sulfate (30-60%) and the acetone fractionation could be eliminated by placing the first ammonium sulfate fractionation (0-45%) on a Sephadex G-200 column. The usefulness of acetone fractionation on the enzyme eluted from the Sephadex column should be investigated. However, further experiments must be performed in order to increase the cell yield without repression of the decarboxylation-dependent transaminase. The low cell yield obtained from cells grown on AIB was the major difficulty in purifying and studying the decarboxylation-dependent transaminase. The use of batch dialysis culture (63) or the use of Dworken and Foster (22) medium may prove advantageous.

The decarboxylation-dependent transaminase was easily resolved into apoenzyme by dialysis in the presence of AIB. It was apparent that the mechanism by which AIB resolved the enzyme was non-enzymatic. Resolution of the enzyme was accomplished at 4 C and it did not require previous incubation of the holoenzyme with the substrate. Since the aldehyde form of

the coenzyme bound more tightly to the apoenzyme than the amino form, a lysine residue of the protein was believed to be involved in the binding. If a Schiff base was formed between the coenzyme and the epsilon amino group of lysine, resolution in the presence of AIB was visualized as a transaldimination reaction. The fact that dialysis of the enzyme against Tris buffer (2) also resolved the enzyme while dialysis against phosphate buffer did not, indicated that an amino group was required for resolution, and provided further evidence that a transaldimination reaction probably occurred. The efficiency of the resolution in the presence of different amino acids and amines should be investigated as these experiments may provide some information on the steric conformation of the active site of the enzyme.

It had been well established that the products of AIB degradation were CO_2 and acetone. Stoichiometric amounts of pyruvate and catalytic amounts of PLP were prerequisites for CO_2 and acetone formation. With this information in mind three possible mechanisms could be postulated for the enzymatic reaction. One mechanism would be the direct decarboxylation-dependent transamination between pyruvate and AIB. Theoretically the amino group of AIB could form a Schiff base with the carbonyl group of pyruvate so that the decar-

boxylation-dependent transamination reaction could proceed without direct involvement of PLP. PLP in this case could be involved in maintaining the structural integrity of the enzyme. If PLP functioned only as a quaternary structural component of the enzyme, different activity peaks should be obtained when resolved enzyme was placed on sucrose density gradients in the presence and absence of PLP. However, the results of this experiment showed identical peaks for the enzyme preparations and provided evidence against this possibility. Furthermore, reduction of the cofactor to the apoprotein would not necessarily alter the enzyme activity when assayed in the presence and absence of PLP. As in phosphorylase (25) the reduced holoenzyme should also have the same activity as the reduced apoenzyme. The results obtained in this study indicated that PLP was a functional coenzyme and not a structural component alone. However, further purification of the decarboxylation-dependent transaminase is required to determine if PLP possesses a dual function as in tryptophanase (53) and L-aspartate β-decarboxylase (71). PLP is required for the function and the proper quaternary structure of these well-studied enzymes. The results further indicated that the amino or aldehyde moiety of the coenzyme were involved in the reaction. The ability of PMP to replace PLP also

supported this idea. It was apparent that the direct decarboxylation-dependent transamination between AIB and pyruvate did not occur.

A second possible mechanism for the enzymatic degradation of AIB was the formation of ISPA, which would subsequently transaminate with pyruvate. The decarboxylation-dependent transamination reaction could be carried out by one or two enzymes. The inability to detect CO_2 or ISPA in the absence of pyruvate could be explained if ISPA or acetone were inhibitors of the decarboxylation reaction. If ISPA was a product, inhibition should have taken place at very low concentration of this compound because ISPA could not be detected during the enzymatic reaction. On the other hand, if acetone was an inhibitor, then higher concentrations would be required to inhibit the decarboxylation reaction because the ketone is easily detected during the reaction. The inability to detect any acetone or ISPA inhibition of the decarboxylation-dependent transaminase and the inability to detect any transamination between ISPA and pyruvate indicated that this mechanism was inoperative in the reaction.

The third possible mechanism for the enzyme was the decarboxylation-dependent transamination proposed by Kalyankar and Snell (37) for degradation of α-dialkyl amino acids in PL model systems. The pyruvate

requirement for the enzymatic decarboxylation-dependent transamination reaction was to regenerate the PLP form of the coenzyme. The ease with which the holoenzyme resolved in the presence of substrate may indicate that one or two enzymes could be involved in the overall reaction. In the presence of PLP one enzyme could decarboxylate AIB to CO_2 and acetone with the resulting formation of PMP. PMP could then be transaminated with pyruvate by another enzyme. However, no evidence of PMP-pyruvate transamination was obtained. On the other hand one mole of enzyme in the PMP form should transaminate one mole of pyruvate per active site. The resulting PLP form of the enzyme would be inactive for further transamination in the same direction. The results of the dialysis experiment presented in Figure 8 showed that PMP-pyruvate transamination did not occur in stoichiometric manner with enzyme in the absence of AIB. The experiment was designed so that if transamination between pyruvate and PMP occurred, the enzyme would be in the PLP form, would not be resolved, and PLP would not be required for decarboxylation-dependent transamination activity. It was also evident that the enzyme could not catalyze the decarboxylation of AIB in the absence of pyruvate. In order to determine decarboxylation of AIB in stoichiometric manner with enzyme in the absence of the keto acid, labeled

substrate must be used. Experiments conducted using AIB-1C^{14} at different enzyme concentration in the absence of pyruvate were inconclusive and it was not known if this reaction took place. If no decarboxylation of AIB occurred using substrate amounts of enzyme a strict control mechanism was evident. That is the enzyme would not decarboxylate or transaminate in the absence of one of the substrates. The fact that AIB and pyruvate increased PMP and PLP binding, respectively, indicated that these compounds may be involved in some function other than simply as substrates. The binding of AIB and pyruvate perhaps altered the enzyme configuration to one that was compatible with enzymatic activity. The kinetic data presented here indicated that both substrates were allosteric effectors for the enzyme. If the enzyme was an allosteric enzyme, AIB and pyruvate acted as modulators and this would explain the inability of the enzyme to act on either substrate in the absence of the other. However, since the enzyme was not in a homogeneous state one must cautiously interpret the kinetic data. It was interesting to note that Hill plots of the kinetic data gave slope values greater than 2, but these values may only indicate the interaction of two components in the system. Since the enzyme definitely required two substrates for activity, the Hill plots

may be meaningless.

Evidence for the oxidation of acetone to acetol by bacteria was provided by Levine and Krampitz (41). Lukins (44) later demonstrated acetol accumulation from acetone-grown cells, and this compound was believed to be broken down to a C2 plus a C1 unit (59, 41). There is also evidence in the literature for the existence of methyl ketone oxygenases in cells of aerobic pseudomonad (26, 27).

The data obtained from oxidation studies with whole cells on acetone, acetol, pyruvic aldehyde and methyl acetate indicated that these compounds may be involved in the catabolism of AIB.

The data presented here indicated that pyruvate may be formed from AIB-grown cells via pyruvic aldehyde as this compound was only oxidized by cells grown on AIB, acetone or acetol. This pathway may be used to provide a source of pyruvate which was required for the decarboxylation-dependent transaminase. Theoretically, only a small amount of pyruvate would be required in cells grown on AIB, provided the organism contained alanine dehydrogenase. The presence of this enzyme would regenerate pyruvate from the alanine formed and maintain a constant supply of the keto acid. As pyruvate was not oxidized by AIB, acetone or acetol-grown cells the organism may obtain its energy from

acetone via MeAc or from acetol via acetate. The inability of acetate, pyruvate and glycerol-grown cells to oxidize MeAc indicated the presence of an inducible esterase. The small oxidation of MeAc by acetol-grown cells indicated that acetol or acetone contamination in the acetol may induce an esterase. In order to further elucidate the pathway proposed, the intermediates must be identified in culture fluid or in isolated enzyme-catalyzed reactions. Also cellular extracts must provide enzymatic evidence for the breakdown of the intermediates.

LITERATURE CITED

1. Aaslestad, H. G. 1965. The bacterial catabolism of 2-methylalanine; Characteristics of the enzyme 2-methylalanine decarboxylase. Ph.D. Dissertation. Louisiana State University: 111 p. Baton Rouge, La.

2. Aaslestad, H. G., P. J. Bouis, Jr., A. T. Phillips, and A. D. Larson. 1968. Characterization of a decarboxylation-dependent transaminase, p. 479. In E. E. Snell, A. E. Braunstein, E. S. Severin, and Yu. M. Torchinsky (ed.), Pyridoxal catalysis: Enzymes and model systems. Interscience Publishers, New York.

3. Aaslestad, H. G., and A. D. Larson. 1964. Bacterial metabolism of 2-methylalanine. J. Bacteriol. 88:1296-1303.

4. Bailey, G. B., and W. B. Dempsey. 1967. Purification and properties of an α-dialkyl amino acid transaminase. Biochem. 6:1526-1533.

5. Bailey, G. B., and W. B. Dempsey. 1968. Purified decarboxylation-dependent transaminase, p. 491. In E. E. Snell, A. E. Braunstein, E. S. Severin, and Yu. M. Torchinsky (ed.), Pyridoxal catalysis: Enzymes and model systems. Interscience Publishers, New York.

6. Bouis, Jr., P. J. 1967. Studies of a decarboxylation-dependent transaminase from a soil pseudomonad. MS thesis. Louisiana State University. Baton Rouge, La.

7. Braunstein, A. E., R. M. Azarkh, and Z. G. Mogilevskaya. 1956. The inhibitive effects of hydroxylamine and α-methyl-DL-glutamic acid on the reaction rate of enzymatic transamination. Chem. Abs. 50:1945.

8. Braunstein, A. E. 1960. Pyridoxal phosphate, p. 113. In P. D. Boyer, H. Lardy, and K. Myrbäck (ed.), The enzymes. Academic Press, New York.

9. Buffoni, F. 1966. Pyridoxal catalysis in pig plasma benzylamine oxidase (histaminase), p. 363. In E. E. Snell, A. E. Braunstein, E. S. Severin, and Yu. M. Torchinsky (ed.), Pyridoxal catalysis: Enzymes and model systems. Interscience Publishers, New York.

10. Carlsson, A., and M. Lindqvist. 1962. In-vitro decarboxylation of α-methyl DOPA and α-methylmetatyrosine. Acta. Phsyiol. Scand. 54:87-94.

11. Christensen, H. N., A. J. Aspen, and E. G. Rice. 1956. Metabolism in the rat of three amino acids lacking the alpha hydrogen. J. Biol. Chem. 220:287-294.

12. Christensen, H. N., T. R. Riggs, H. Fischer, and I. M. Palatine. 1952. Amino acid concentration by cell neoplasm: relation among amino acids. J. Biol. Chem. 198:1-15.

13. Christensen, H. N., and T. R. Riggs. 1956. Structural evidence for chelation and Schiff base formation in amino acid transfer into cells. J. Biol. Chem. 220:265-278.

14. Christensen, H. N., and J. C. Jones. 1962. Amino acid transport models: renal resorption and resistance to metabolic attack. J. Biol. Chem. 237:1203-1206.

15. Clark, W. G. 1963. Inhibition of amino acid decarboxylases, p. 315. In R. M. Hochster, and J. H. Quastel (ed.), Metabolic inhibitors, vol. 1. Academic Press, Inc. New York.

16. Davis, B. J. 1964. Disc electrophoresis. II. Methods and application to human serum proteins. Ann. New York Acad. Sci. 121:404-427.

17. Dempsey, W. B. 1969. Metabolism of α-amino-isobutyric acid by soil bacteria. J. of Bacteriol. 97:182-185.

18. Dempsey, W. B., and E. E. Snell. 1963. Pyridoxamine-pyruvate transaminase. II. Characteristics of the enzyme. Biochem. 2:1414-1419.

19. den Dooren de Jong, L. E. 1926. Bijdrage tot de Kennis van het mineralsatie proces. Dissertation. Rotterdam, The Netherlands, In Stephenson, M. 1949. Bacterial metabolism. 3rd ed. Longmans Green & Co., London.

20. Drapeau, G. R., and P. A. MacLeod. 1963. Sodium dependent active transport of α-amino isobutyric acid into cells of a marine pseudomonad. Biochem. Biophys. Res. Comm. 12: 111-115.

21. Dunathan, H. C. 1966. Conformation and reaction specificity in pyridoxal phosphate enzymes. Proc. Natl. Acad. Sci. (U.S.). 55:712-716.

22. Dworkin, M., and J. W. Foster. 1958. Experiments with some microorganisms which utilize ethane and hydrogen. J. of Bacteriol. 75: 592-603.

23. Ehrlich, F. 1908. Über die spaltung reacemischer amino säüren mittels hefe. Biochem. Zeit. 8:438-466.

24. Fasella, P. 1967. Pyridoxal phosphate, p. 185. In P. D. Boyer, A. Meister, R. L. Sinsheimer, and E. E. Snell (ed.), Annual review of Biochemistry, vol. 36. Annual Reviews, Inc., California.

25. Fischer, E. H., A. W. Forrey, J. L. Hedrick, R. C. Hughes, A. B. Kent, and E. G. Krebs. 1963. Pyridoxal-5'-phosphate in the structure and function of phosphorylase, p. 543. In E. E. Snell, P. M. Fasella, A. Braunstein, and A. R. Fanelli. Chemical and biological aspects of pyridoxal catalysis. The Macmillan Co., New York.

26. Forney, F. W., and A. J. Markovetz. 1969. An enzyme system of aliphatic methyl ketone oxidation. Biochemical and Biophysical Research Communications. 37:31-38.

27. Forney, F. W., and A. J. Markovetz. 1970. Subterminal oxidation of aliphatic hydrocarbons. J. of Bacteriol. 102:281-282.

28. Frieden, E., L. T. Hsu, and K. Dettmer. 1951. Enzymatic degradation of amino acid antagonists. J. Biol. Chem. 192:425-432.

29. Greenberg, L. A., and D. Lester. 1944. A micromethod for the determination of acetone and ketone bodies. J. Biol. Chem. 154:177-190.

30. Hall, P. R., and K. B. Eik-Nes. 1962. Interstitial cell stimulating hormone and penetration of D-xylose-1-C^{14} and α-amino isobutyric acid-1-C^{14} into slices of testes. Proc. Soc. Exptl. Biol. Med. 110:148-151.

31. Hayaishi, O., Y. Nishizuka, M. Tatibana, M. Takeshita, and S. Kuno. 1961. Enzymatic studies on the metabolism of β-alanine. J. Biol. Chem. 236:781-790.

32. Heyl, D., E. Luz, S. A. Harris, and K. Folkers. 1948. Chemistry of vitamin B6. VII. Pyridoxylidene- and pyridoxylamines. J. Am. Chem. Soc. 70:3669-3671.

33. Heyl, D., E. Luz, S. A. Harris, and K. Folkers. 1948. Pyridoxylamines. J. Am. Chem. Soc. 70:1670-1671.

34. Hughes, R. C., W. T. Jenkins, E. H. Fischer. 1962. The site of binding of pyridoxal-5'-phosphate to heart glutamic-aspartic transaminase. Proc. Natl. Acad. Sci. 48:1615-1618.

35. Jencks, E. P., and E. Cordes. 1963. Transaldimination reactions of pyridoxal and related compounds, p. 57. In E. E. Snell, P. M. Fasella, A. Braunstein, and A. R. Fanelli (ed.), Chemical and biological aspects of pyridoxal catalysis, The Macmillan Company, New York.

36. Jenkins, W. T., and I. W. Sizer. 1957. Glutamic aspartic transaminase. J. Am. Chem. Soc. 79:2655-2656.

37. Kalyankar, G. D., and E. E. Snell. 1962. Pyridoxal-catalyzed decarboxylation of amino acids. Biochem. 1:594-600.

38. Katunuma, N., S. Matsuda, and M. Izumi. 1962. Glutamic pyruvic transaminase. Symposia on Enzyme Chemistry. 16:70-77.

39. Kosower, E. M. 1962. Molecular biochemistry. McGraw-Hill Book Company, Inc., New York. p. 118-125.

40. Leighty, J. A., and R. C. Corley. 1937. Amino acid catabolism: the fat of certain synthetic ∝-amino acids administered by subcutaneous injection to the normal dog. J. Biol. Chem. 120:331-334.

41. Levine, S., and L. O. Krampitz. 1952. The oxidation of acetone by a soil diptheroid. J. Biol. Chem. 64:645-650.

42. Longenecker, J. B., M. Ikawa, and E. E. Snell. 1957. The cleavage of ∝-methylserine and ∝-methylolserine by pyridoxal and metal ions. J. Biol. Chem. 226:663-666.

43. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193:265-275.

44. Lukins, H. B. 1962. On the utilization of hydrocarbons, methyl ketones and hydrogen by Mycobacteria. Dissertation. University of Texas. Austin, Texas.

45. Mahler, H. R., and E. H. Cordes. 1966. Biological chemistry. Harper & Row Publishers, New York. p. 672.

46. Malakhova, E. A., and Yu. M. Torchinsky. 1965. Isolation of a coenzyme-quasisubstrate complex from aspartate-glutamate transaminase. Doklady Biochemistry. Proceedings of the Academy of Sciences of the USSR. Biochemistry Section. 161:100-102.

47. Mandeles, S., R. Koppelman, and M. E. Hanke. 1954. Deuterium studies on the mechanism of enzymatic amino acid decarboxylation. J. Biol. Chem. 209:327-336.

48. Marquis, R. E., and P. Gerhardt. 1964. Respiration and passive uptake of α-aminoisobutyric acid, a metabolically inert transport analogue by Bacillus megaterium. J. Biol. Chem. 239:3361-3371.

49. Matsuo, Y. 1957. Formation of a Schiff base of pyridoxal phosphate. Reaction with metal ions. J. Am. Chem. Soc. 79:2011-2015.

50. Matsuo, Y. 1957. Pyridoxal catalysis of non-enzymatic transamination of ethanol solution. J. Am. Chem. Soc. 79:2016-2019.

51. Metzler, D. E., M. Ikawa, and E. E. Snell. 1954. A general mechanism for vitamin B6-catalyzed reactions. J. Am. Chem. Soc. 76:648-652.

52. Metzler, D. E. 1957. Equilibria between pyridoxal and amino acids and their imines. J. Am. Chem. Soc. 79:485-490.

53. Morino, Y., and E. E. Snell. 1967. The subunit structure of tryptophanase. I. The effect of pyridoxal phosphate on the subunit structure and physical properties of tryptophanase. J. Biol. Chem. 242:5591.

54. Morino, Y., and T. Watanabe. 1969. Primary structure of pyridoxal phosphate binding site in the mitochondrial and extra-mitochondrial aspartate aminotransferases from pig heart muscle. Chymotryptic peptides. Biochem. 8:3412-3417.

55. Noall, M. W., T. R. Riggs, L. M. Walker, and H. N. Christensen. 1957. Endocrine control of amino acid transfer distribution of an unmetabolizable amino acid. Science. 126: 1002-1005.

56. Pfister, K., W. J. Leanza, J. P. Conbere, H. J. Becker, A. R. Matzuk, and E. F. Rogers. 1955. α-methyl-α-amino acids. I. Homologs of glutamic acid, methionine, and diaminopimelic acid. J. Am. Chem. Soc. 77:697-700.

57. Roberts, E. 1953. Further studies on inhibition of bacterial glutamic decarboxylases. J. Biol. Chem. 202:359-367.

58. Rosett, T. 1965. Cooling device for use with a sonic oscillator. Appl. Microbio. 13:254-256.

59. Sakami, E. 1950. Formation of formate and labile methyl groups from acetone in the intact rat. J. Biol. Chem. 187:369.

60. Schirch, L. G., and M. Mason. 1963. Serine transhydroxymethylase. J. Biol. Chem. 238: 1032-1037.

61. Schlenk, F., and A. Fischer. 1945. Note on the purification and properties of glutamic-aspartic transaminase. Arch. of Biochem. 8:337-338.

62. Schlenk, F., and A. Fischer. 1947. Studies on glutamic-aspartic acid transaminase. Arch. of Biochem. 12:69-78.

63. Schultz, J. S., and P. Gerhardt. 1969. Dialysis culture of microorganisms: design, theory, and results. Bact. Reviews. 33:1-47.

64. Snell, E. E. 1945. The vitamin B6 group. The reversible interconversion of pyridoxal and pyridoxamine by transamination reaction. J. Am. Chem. Soc. 67:194-197.

65. Snell, E. E., and J. C. Rabinowitz. 1948. The microbiological activity of pyridoxal-amino acids. J. Am. Chem. Soc. 70:3432-3434.

66. Snell, E. E., P. M. Fasella, A. Braunstein, and A. R. Fanelli (ed.). 1963. Chemical and biological aspects of pyridoxal catalysis. The Macmillan Co., New York.

67. Snell, E. E., A. E. Braunstein, E. S. Severin, and Yu. M. Torchinsky (ed.). 1968. Pyridoxal catalysis: enzymes and model systems. Interscience Publishers, New York.

68. Sourkes, T. L. 1954. Inhibition of dehydroxyphenylalanine decarboxylase by derivatives of phenylalanine. Arch. Biochem. 51:444-456.

69. Stanier, R. Y. 1947. Simultaneous adaption: a new technique for the study of metabolic pathways. J. Bacteriol. 54:339-348.

70. Strausbauch, P. H., and E. H. Fischer. 1970. Structure of the binding site of pyridoxal-5'-phosphate to E. coli glutamate decarboxylase. Biochem. 9:233-238.

71. Tate, S. S., and A. Meister. 1969. The effects of various vitamin B6 5'-phosphate derivatives on the structure of L-aspartate β-decarboxylases. Biochem. 8:1056-1065.

72. Thibert, R. J., J. F. G. Duderich, and G. W. Kosicki. 1967. Behavior of α substituted cystines in a cystathionase system and in a pyridoxal phosphate model system. Canad. J. of Biochem. 45:1595-1617.

73. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric techniques. 4th ed. Burgess Publishing Co., Minneapolis 15, Minnesota.

74. Umbreit, W. W. 1955. Reactions of α-methyl amino acids, p. 48. In W. D. McElroy, and H. B. Glass (ed.), A symposium on amino acid metabolism. The John Hopkins Press, Baltimore.

75. Underwood, J. C., and L. B. Rockland. 1954. Small scale filter paper chromatography factors affecting the separation and sequence of amino acids. Anal. Chem. 26:1553-1557.

76. Wada, H., and E. E. Snell. 1962. Enzymatic transamination of pyridoxamine I with oxaloacetate and α-ketoglutarate. J. Biol. Chem. 237:127-137.

77. Warburg, O., and W. Christian. 1942. Isolierung und kristallisation des garungsferments enolase. Biochem. Z. 310:384-421.

78. Weissbach, H., W. Lovenberg, and S. Undenfriend. 1960. Enzymatic decarboxylation of α-methyl amino acids. Biochem. Biophys. Res. Comm. 3:225-227.

79. Wilson, E. M., and E. E. Snell. 1962b. Metabolism of ∝-methylserine. II. Stereospecificity of ∝-methylserine hydroxymethyl-transferase. J. Biol. Chem. 237:3180-3184.

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Major Field: Microbiology

Title of Thesis: Characterization of a Decarboxylation-dependent Transaminase and the Metabolism of Alpha-aminoisobutyric Acid.

Approved:

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